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Customer No.:	21559		
Title:	FUSION PROTEIN THAT IMPARTS SELECTIVE PROLIFERATION ACTIVITY		

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DECLARATION OF YASUJI UEDA UNDER 37 C.F.R. § 1.132

I declare:

1. I am a co-inventor of the subject matter that is described and claimed in the above-referenced application. I am currently an Assistant Professor at the Chiba University Graduate School of Medicine. I have over 20 years technical experience in the fields of Molecular Biology, Cell Biology, Immunology, etc. A copy of my curriculum vitae is attached as Appendix A.

2. G-CSF receptors are a class of proteins that were extraordinarily well characterized at the time the application was filed. The structural features, including the Immunoglobulin-like domain, the cytokine receptor homologous domain, the three fibronectin type III domains, and the intracellular domain, defining this class of receptors were also known. For instance, Fukunaga et al. (Cell 61:341-350, 1990; copy enclosed as Exhibit A) describes the murine G-CSF receptor sequence and notes that the sequence is highly homologous to that of the human G-CSF receptor. Larsen et al. (J. Exp. Med. 172:1559-1570, 1990; copy enclosed as Exhibit B) describes the human G-CSF receptor sequence. In addition, Fukunaga et al. (EMBO J.: 10:2855-2865, 1991;

copy enclosed as Exhibit C) describes functional domains of human and mouse G-CSF receptors. In view of the knowledge in the art at the time the application was filed, a skilled artisan would readily recognize a G-CSF receptor sequence.

3. Estrogen receptors are part of a conserved and well-characterized family of proteins. The sequences of various members of the estrogen receptor family were known at the time the application was filed. For instance, the human estrogen receptor sequence was disclosed in a 1986 publication (see Greene et al., Science 231:1150-1154, 1986; copy enclosed as Exhibit D). Given that the structure of the estrogen receptor and of its estrogen-binding domain was known at the time the application was filed, one skilled in the art would readily recognize whether a given sequence is that of an estrogen receptor. Moreover, a skilled artisan, at the time of filing, would have recognized an estrogen-binding domain of an estrogen receptor.

4. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

December 13, 2006
Date

Gary W
Yasuji Ueda

CURRICULUM VITAE OF DR. YASUJI UEDA

Name: Yasuji Ueda

Contact Information:

Address: 1-8-1, Inohana, Chuo-ku, Chiba-shi, Chiba, Japan

Telephone no.: +81-43-226-2965

E-mail address: y_ueda@faculty.chiba-u.jp

Current Post: Assistant Professor at Chiba University Graduate School of Medicine

Education:

1978 – 1982 B.S., Faculty of Science,
Kyoto University, Kyoto, Japan

1982 – 1986 D.S., Faculty of Science,
Kyoto University, Kyoto, Japan

Professional Experience:

1986 – 1996 Assistant Professor at Tokyo University of Science

1996 – 2004 Research Scientist at DNAVEC Research Inc.

2004 – 2004 Research Manager at DNAVEC Corporation

2004 - Present Position

Fields of Research:

Cell Biology

Molecular Biology

Immunology

List of publications:

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Expression Cloning of a Receptor for Murine Granulocyte Colony-Stimulating Factor

Rikiro Fukunaga, Etsuko Ishizaka-Ikeda,
Yoshiyuki Seto, and Shigekazu Nagata
Osaka Bioscience Institute
6-2-4 Furuedai
Suita-shi
Osaka 565
Japan

Summary

Two cDNAs encoding the receptor for murine granulocyte colony-stimulating factor (G-CSF) were isolated from a CDM8 expression library of mouse myeloid leukemia NFS-60 cells, and their nucleotide sequences were determined. Murine G-CSF receptor expressed in COS cells could bind G-CSF with an affinity and specificity similar to that of the native receptor expressed by mouse NFS-60 cells. The amino acid sequence encoded by the cDNAs has demonstrated that murine G-CSF receptor is an 812 amino acid polypeptide (M_r 90,814) with a single transmembrane domain. The extracellular domain consists of 601 amino acids with a region of 220 amino acids that shows a remarkable similarity to rat prolactin receptor. The cytoplasmic domain of the G-CSF receptor shows a significant similarity with parts of the cytoplasmic domain of murine interleukin-4 receptor. A 3.7 kb mRNA coding for the G-CSF receptor could be detected in mouse myeloid leukemia NFS-60 and WEHI-3B D⁺ cells as well as in bone marrow cells.

Introduction

Production of hematopoietic cells is regulated by hormone-like growth and differentiation factors called colony-stimulating factors (CSFs). CSFs include granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and interleukin-3 (IL-3) (Metcalf, 1989; Nicola, 1989). G-CSF, produced mainly by macrophages, is important in regulating blood levels of neutrophils and in activating mature neutrophils. G-CSF stimulates some myeloid leukemia cells to proliferate or to differentiate into neutrophilic granulocytes (Nagata, 1990).

Murine G-CSF and human G-CSF have been purified (Nicola et al., 1983; Nomura et al., 1986), and cDNAs encoding G-CSF have been molecularly cloned (Nagata et al., 1986a, 1986b; Souza et al., 1986; Tsuchiya et al., 1986). Human G-CSF is a 174 amino acid polypeptide, while murine G-CSF consists of 178 amino acids. Human and mouse G-CSFs are highly homologous (72.6%) at the amino acid sequence level, in agreement with the lack of species specificity between them (Nicola et al., 1985). Although the primary structure of G-CSF does not show homology with other CSFs or growth factors, it has a weak similarity with interleukin-6 (IL-6), which stimulates pro-

liferation and differentiation of B-lymphocytes (Nagata, 1990). Human G-CSF produced by recombinant DNA technology has proven to be a potent regulator of neutrophils in vivo using animal model systems (Tsuchiya et al., 1987; Nicola, 1989). Recent clinical trials in patients suffering from a variety of hemopoietic disorders have shown that the administration of G-CSF is beneficial in chemotherapy and bone marrow transplantation therapy (Morstyn et al., 1989).

Despite the biological importance of G-CSF, little is known about the mechanism of G-CSF-induced signal transduction in the proliferation and differentiation of neutrophilic granulocytes. Several reports in human and mouse systems have suggested that the expression of the G-CSF receptor is restricted to progenitor and mature neutrophils and various myeloid leukemia cells (Nicola and Metcalf, 1984, 1985; Nicola et al., 1985; Begley et al., 1987; Park et al., 1989). However, the G-CSF receptor has also recently been found in nonhemopoietic cells, such as human endothelial cells (Bussolino et al., 1989) and placenta (Uzumaki et al., 1989). Biochemical characterization of the G-CSF receptor has been hampered by the low number of receptors present on the cell surface (at most 1000–2000 receptors per cell). A limited number of studies have indicated that cells of the neutrophilic lineage have a single class of binding sites for G-CSF with an equilibrium dissociation constant of 100–500 pM (Nicola and Metcalf, 1984; Park et al., 1989; Uzumaki et al., 1989). Cross-linking studies of the receptor with the radiolabeled G-CSF have suggested a M_r of 150,000 for the mouse G-CSF receptor in WEHI-3B D⁺ cells (Nicola and Peterson, 1986). Recently, we were able to solubilize mouse G-CSF receptor in an active form from NFS-60 cells and succeeded in purifying the receptor as a protein with a M_r of 100,000–130,000 (R. F., E. I., and S. N., unpublished data).

In this work, we isolated cDNAs encoding the murine G-CSF receptor from mouse myeloid leukemia NFS-60 cells. When transfected into COS cells, the cDNA directed expression of a receptor that has similar properties to that of the native G-CSF receptor on NFS-60 cells. The amino acid sequence of the G-CSF receptor indicates that it belongs to the recently identified growth factor receptor family (Bazan, 1989).

Results

Expression Cloning of the G-CSF Receptor cDNA

To isolate the cDNA coding for the G-CSF receptor, we used a COS cell expression system developed to isolate the murine erythropoietin receptor (D'Andrea et al., 1989). Double-stranded cDNA was synthesized using mRNA from mouse myeloid leukemia NFS-60 cells, which have relatively higher expression of the G-CSF receptor than other G-CSF-responsive myeloid leukemia cells, such as WEHI-3B D⁺ or 32DC13. A cDNA library was constructed in the mammalian expression vector CDM8 (Seed, 1987) as 884 pools of 60–80 clones. Plasmid DNAs from each

pool were prepared by the boiling method and introduced into COS-7 cells grown in 6-well microtiter plates. At 72 hr posttransfection, binding reactions of ^{125}I -G-CSF (1.7×10^5 cpm [200 pM] in a 0.6 ml volume) to COS cells were carried out at 37°C for 2 hr instead of 4°C in order to obtain a greater signal (D'Andrea et al., 1989). Under these conditions, the background binding of labeled G-CSF to transfected or untransfected COS cells was routinely 308 ± 38 (SD) cpm. Plasmid DNAs from two pools (I62 and J17) yielded binding of 500 cpm and 912 cpm of ^{125}I -G-CSF, respectively, when transfected into COS-7 cells. The bacterial clones of pools I62 and J17 were arranged in 12 subgroups of 12 clones each and assayed as above. Some subgroups gave positive responses, that is, binding of 3710–4010 cpm of ^{125}I -G-CSF to COS cells. By assaying single clones from each positive subgroup, two independent clones (pI62 and pJ17) were identified. When plasmid DNAs from pI62 and pJ17 were transfected into COS-7 cells, the binding assay gave values of 30,300 cpm and 31,600 cpm, respectively.

Binding Characteristics of the Cloned Receptor

The binding characteristics of the G-CSF receptor expressed on COS cells were examined. COS cells transfected with the plasmid CDM8 or pJ17 were incubated at 4°C for 4 hr with various concentrations of ^{125}I -G-CSF in the presence or absence of at least a 500-fold excess of unlabeled G-CSF (800 nM). Untransfected COS cells or COS cells transfected with the CDM8 vector alone did not show any significant specific binding of ^{125}I -G-CSF. On the other hand, labeled G-CSF was bound at 4°C to the COS cells transfected with the plasmid pJ17. As shown in Figure 1, a Scatchard analysis of the specific binding of ^{125}I -G-CSF to COS cells revealed a single species of binding site with an equilibrium dissociation constant of 290 pM and 3.0×10^4 receptors per cell. If the transfection efficiency of COS cells was assumed to be 10%–20% (Sompayrac and Danna, 1981), the positively transfected COS cells probably expressed the recombinant G-CSF receptor at 1.5 – 3.0×10^5 molecules per cell. Since the native G-CSF receptor on NFS-60 cells has an equilibrium dissociation constant of 180 pM (Figure 1D), these results suggest that the polypeptide coded by the cDNA in the plasmid pJ17 is sufficient to express the high-affinity receptor for murine G-CSF.

Human G-CSF competes with mouse G-CSF for binding to mouse WEHI-3B D $^+$ cells (Nicola et al., 1985). Accordingly, an excess of unlabeled recombinant human G-CSFs produced either by mammalian cells or *Escherichia coli* could compete well with labeled mouse G-CSF for binding to COS cells transfected with the plasmid pJ17 (Figure 2). No inhibition of binding of ^{125}I -G-CSF to COS-7 cells was observed in the presence of unlabeled recombinant murine GM-CSF, murine IL-3, murine IL-6, murine leukemia inhibitory factor (LIF), rat prolactin, or human M-CSF. These results correlated well with the specificity of the native G-CSF receptor or the purified receptor on NFS-60 cells (R. F., E. I., and S. N., unpublished data).

Previously, we observed that the G-CSF receptor purified from NFS-60 cells has a M_r of 100,000–130,000. To

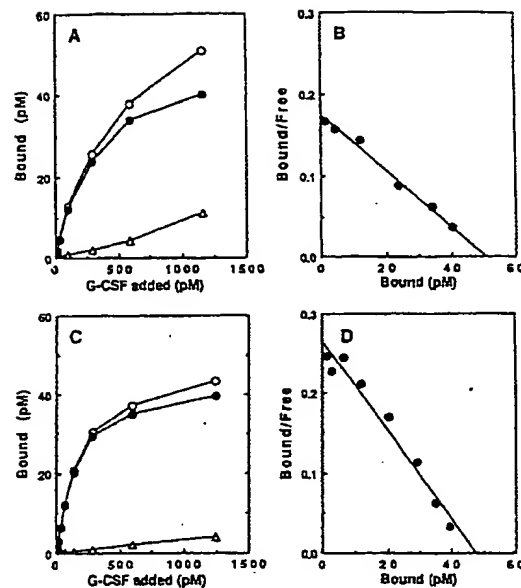


Figure 1. Binding of Radiolabeled G-CSF to COS Cells Expressing the Recombinant G-CSF Receptor and NFS-60 Cells

(A) Saturation binding of ^{125}I -G-CSF to COS cells. COS cells (1×10^6) transfected with the plasmid pJ17 were incubated with various amounts of ^{125}I -G-CSF with or without an excess of unlabeled G-CSF as described in the Experimental Procedures. The specific binding (●) is shown as the difference between total (O) and nonspecific (Δ) binding. (B) Scatchard plot of G-CSF binding data in COS cells. (C) Saturation binding of ^{125}I -G-CSF to NFS-60 cells. Total (O), non-specific (Δ), and specific (●) binding to cells are shown. (D) Scatchard plot of G-CSF binding data in NFS-60 cells.

determine the molecular size of the recombinant G-CSF receptor expressed in COS cells, chemical cross-linking of the receptor with ^{125}I -G-CSF was carried out. As shown in Figure 3, cross-linking of the G-CSF receptor on NFS-60 cells with labeled mouse G-CSF (M_r , 25,000) yielded a band with an apparent M_r of 125,000–155,000 (lane 6), indicating that the M_r of the murine G-CSF receptor on NFS-60 cells is 100,000–130,000. Similarly, cross-linking of ^{125}I -mouse G-CSF to the receptor expressed in COS cells gave a major band of M_r 120,000–150,000 (lane 4), which is slightly smaller than that detected in NFS-60 cells. These bands were not observed when the cross-linking experiment was carried out in the presence of 1.5 μM unlabeled G-CSF (lanes 2 and 5) or when the cross-linking agents were omitted (lane 3). The slightly different M_r observed in COS cells and NFS-60 cells may be explained by the differential glycosylation in these cell lines.

The Structure of Murine G-CSF Receptor

Digestions of the plasmid pJ17 and pI62 with XhoI released cDNA inserts of 3.2 kb and 3.0 kb, respectively. As shown in Figure 4A, the restriction maps of these inserts were identical except that the 5' terminus of clone pI62 is 81 bp longer than that of pJ17 and the 3' terminus of the clone pJ17 is 238 bp longer than that of pI62. When

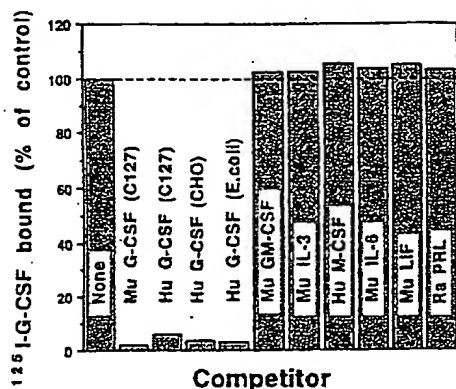


Figure 2. Specificity of G-CSF Binding to Recombinant G-CSF Receptor Expressed in COS Cells

COS cells transfected with the cDNA for the G-CSF receptor (pJ17) were incubated with 2 ng of 125 I-mouse G-CSF in the absence or presence of 1 μ g of unlabeled murine G-CSF, human G-CSF, murine GM-CSF, human M-CSF, murine IL-6, murine LIF, or rat prolactin. As human G-CSF, human recombinant G-CSFs produced in mouse C127 cells, in Chinese hamster ovary cells, or in *E. coli* were used. The radioactivities bound to COS cells in each experiment are expressed as a percentage of that obtained without competitor.

the cDNAs were sequenced, the two sequences were found to be identical within the overlapping region. Although the two cDNAs contained the complete coding sequence for the G-CSF receptor, they contained neither the poly(A) tract nor the poly(A) addition signal. The cDNA library was, therefore, rescreened by colony hybridization using the 2.5 kb HindIII-XbaI fragment of pJ17 as a probe. Fifteen positive clones were obtained from about 60,000 clones, and one of them (pF1) had 603 bp of 3' noncoding region and contained two overlapping poly(A) addition signals. The composite nucleotide sequence of the three cloned cDNAs (pI62, pJ17, and pF1) is presented in Figure 5 together with the predicted amino acid sequence. There is a long open reading frame starting from the initiation codon ATG at nucleotide positions 180-182 and ending at the termination codon TAG at positions 2691-2693. The open reading frame (2511 nucleotides) can code for a protein consisting of 837 amino acids, including the NH_2 -terminal methionine. In the 5' sequence upstream of the

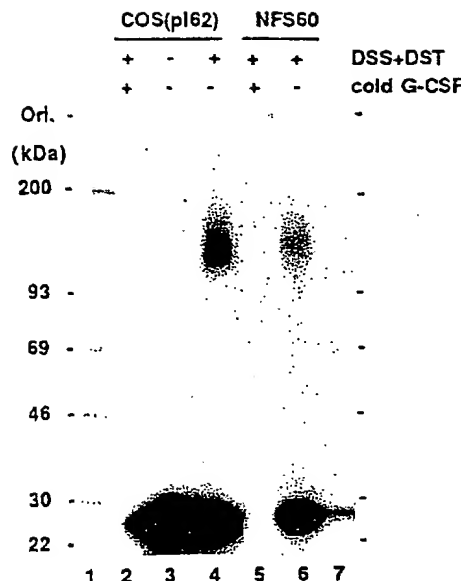


Figure 3. Chemical Cross-Linking of the G-CSF Receptor Expressed in COS and NFS-60 Cells

COS cells (5.2×10^5 cells/lane) transfected with the plasmid pI62 were incubated with 125 I-G-CSF with (lane 2) or without (lanes 3 and 4) an excess of unlabeled murine G-CSF and chemically cross-linked as described in the Experimental Procedures. Mouse NFS-60 cells (3×10^6 cells/lane) were similarly incubated with 125 I-G-CSF with (lane 5) or without (lane 6) an excess of unlabeled G-CSF and cross-linked with DSS and DST. The cell lysate was analyzed by SDS-PAGE on a 4%-20% gradient polyacrylamide gel and exposed to X-ray film at -80°C for 2 days with intensifying screens. As size markers, ^{14}C -labeled molecular weight standards (rainbow marker, Amersham) were electrophoresed in parallel (lanes 1 and 7), and sizes of standard proteins are shown in kd.

long open reading frame, three other potential initiation codon ATGs can be found at positions 73, 105, and 126. All of these are followed by short open reading frames. Deletion of these ATG codons from the cDNA by digesting the plasmid pI62 with HindIII did not increase or decrease the expression level of the recombinant G-CSF receptor in COS cells (R. F. and S. N., unpublished data).

The long open reading frame starts with a stretch of hydrophobic amino acids that seems to serve as a signal se-

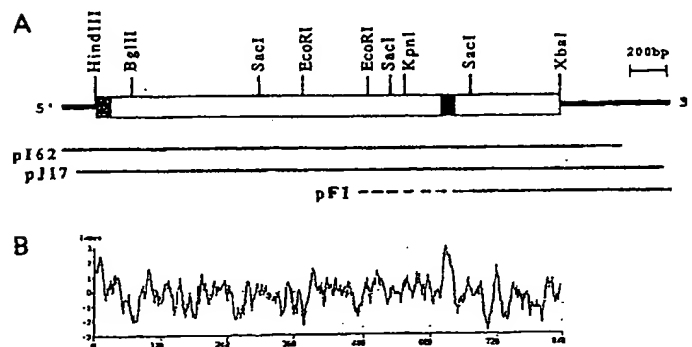


Figure 4. Murine G-CSF Receptor cDNAs

(A) Schematic representation and restriction map of three independent cDNAs (pI62, pJ17, and pF1) for murine G-CSF receptor. The box represents the open reading frame. The stippled and black regions indicate the signal sequence and the transmembrane region, respectively. The cleavage sites for restriction enzymes are shown.

(B) Hydropathy plot of the amino acid sequence of murine G-CSF receptor. The hydropathy plot was obtained by the method of Kyte and Doolittle (1982) using a window of ten residues. The numbers under the plot indicate positions of the amino acid residues of the precursor protein.

TTCCAGGCCAGCCTGGTCTACAAAGTGAGTTCACGACAGCCAGAGCTATACAGAGAAACCTGTCTTGAAAAAAATTAAAGCAGAAAGCTGATTAATAAAGTTTTTTTATAC

quence. By comparing the 5' portion of the sequence with typical signal peptide cleavage sites (von Heijne, 1986), the 26th amino acid (Cys) from the initiation codon was tentatively assigned as the first amino acid of the mature protein. The mature murine G-CSF receptor thus would consist of 812 amino acids with a calculated M_r of 90,814, which is 5,000–35,000 smaller than the M_r (95,000–125,000) estimated from the cross-linking experiment (Figure 3) or the M_r of the purified murine G-CSF receptor (R. F., E. I., and S. N., unpublished data). The difference is probably due to the attachment of sugar moieties to some of the 11 putative N-glycosylation sites (Asn-X-Thr/Ser) found on the extracellular domain of the G-CSF receptor (Figure 5). A hydropathy plot (Kyte and Doolittle, 1982) of the amino acid sequence of the mature G-CSF receptor (Figure 4B) revealed a stretch of 24 uncharged amino acids extending from Leu-602 to Cys-625, which is followed by three basic amino acids. These properties are consistent with those observed in the membrane-spanning segments of many proteins (Sabatini et al., 1982). The mature G-CSF receptor thus appears to consist of an extracellular domain of 601 amino acids, a membrane-spanning domain of 24 amino acids, and a cytoplasmic domain of 187 amino acids. The NH_2 -terminal half of the extracellular domain is abundant in cysteine residues (17 residues in 373 amino acids), which seems to be a feature common to the ligand-binding domain of many receptors (McDonald et al., 1989). As found in the erythropoietin receptor (D'Andrea et al., 1989), the G-CSF receptor is rich in proline (80 residues, 9.9%). Furthermore, the content of tryptophan residues in murine G-CSF receptor is relatively high (26 residues, 3.2%), although they show no particular area of localization within the receptor.

Expression of the G-CSF Receptor mRNA

G-CSF stimulates the proliferation of mouse myeloid leukemia NFS-60 cells, while WEHI-3B D⁺ cells can be induced to differentiate into monocytes and granulocytes by G-CSF (Nagata, 1990). To determine whether the same mRNA is expressed in NFS-60 and WEHI-3B D⁺ cells, Northern hybridization was carried out using the cDNA from plasmid pJ17. As shown in Figure 6, a 3.7 kb mRNA could be detected in RNAs from NFS-60 cells (lanes 2 and 3) as well as from WEHI-3B D⁺ cells (lane 5). The amount of mRNA for the G-CSF receptor is about ten times higher in NFS-60 cells than in WEHI-3B D⁺ cells, which agrees with our observation that NFS-60 cells bind three to four times more ¹²⁵I-G-CSF than WEHI-3B D⁺ cells (unpublished data). In contrast, no transcript for the G-CSF receptor was detected in RNAs from other mouse myeloid leukemia FDC-P1 cells (lane 4), which do not respond to G-CSF, or from nonhemopoietic cell lines such as L929 (lane 1) or C1271 (data not shown). When mRNA expression was examined in various mouse tissues, only bone

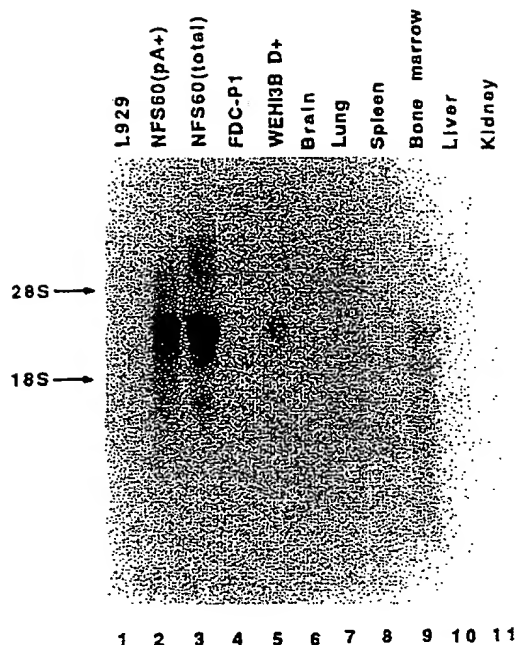


Figure 6. Northern Hybridization Analysis of Murine G-CSF Receptor mRNA

Total RNA or poly(A) RNA was prepared from mouse cell lines: L929 (lane 1), NFS-60 (lanes 2 and 3), FDC-P1 (lane 4), and WEHI-3B D⁺ (lane 5), or from mouse tissues: brain (lane 6), lung (lane 7), spleen (lane 8), bone marrow (lane 9), liver (lane 10), and kidney (lane 11). Total RNA (30 µg) (lanes 1 and 3–11) or 2 µg of poly(A) RNA (lane 2) was electrophoresed on a 1.3% agarose gel containing 6.6% formaldehyde and analyzed by Northern hybridization as described in the Experimental Procedures.

marrow cells gave a signal corresponding to the 3.7 kb mRNA (lane 9). The similar size of the mRNAs observed in bone marrow cells and NFS-60 cells suggests that the authentic mRNA for the G-CSF receptor is expressed in mouse myeloid leukemia NFS-60 cells.

Discussion

The mechanisms of signal transduction induced by various hemopoietic growth and differentiation factors, including G-CSF, are not fully understood, in part because their receptors are expressed in low levels on the cell surface. Recently, receptors for several cytokines and lymphokines have been molecularly cloned by various techniques (Yamasaki et al., 1988; Mosley et al., 1989; Hatakeyama et al., 1989; D'Andrea et al., 1989; Gearing et al., 1989; Itoh et al., 1990). To isolate the cDNA for the G-CSF receptor, we used the expression cloning method developed by D'Andrea et al. (1989). We chose this method since a single

Figure 5. Nucleotide Sequence and Predicted Amino Acid Sequence of the Murine G-CSF Receptor cDNA

Numbers above and below each line refer to the nucleotide position and amino acid position, respectively. Amino acids are numbered starting at Cys-1 of the mature G-CSF receptor. On the amino acid sequence, the signal sequence and the transmembrane domain are underlined. Two overlapping poly(A) addition signals (AATAAA) are also underlined. Potential N-glycosylation sites (Asn-X-Ser/Thr) (11 in the extracellular domain and 2 in the cytoplasmic domain) are boxed.

A

G-CSFR (96) QYFFASSNLSCLMHLLTTNSLVCQWEPGFETHLEPTSPILKSFRSIRADCQY
 PRLR (1) QSPPGKPEIHKCRSPD-KETFTCWNPGTDGGLPTNYSL-----TYSKE
 GHR (27) TNSSKEEKFTKCRSPE-RETFSCHWTDEVHHGTKNLGPLQLFYTRRNTOE
 cons C W (L)

G-CSFR (146) QGDTIPDCVAKKR--QNNCSIPRKNLLLYQYMAIWVOAENMLGSSESPKL
 RPLR (44) GEKTTYECPDYKTSGPNSCFFSKQYTSIWKIYIITVNATNQMGSSSDPL
 GHR (76) WTOEWKECPDYVSAGENSCYFNSSFTSIWIPYCI--KLTSNGGTVDEKCF
 cons C S G

G-CSFR (194) CLDPMDVKLEPFMLOALDIGPDVVSHOPGCLWLSWKPWKPSEYMEOECE
 RPLR (94) YVDVTYIVEPEPPRNLTLEV-KQLK-DKKTYLWVKWSPPTITDVKTGWFT
 GHR (124) SVDET--VOPDPPIALNWTLLNVSLTGIHADIOVRWEAPRNADIQKGWMV
 cons (V) (P) W

G-CSFR (244) LRYQPQLK--GANWTLVFHLPSSKDOFELCGLHQAPVYTLOMRCIRSSL
 RPLR (142) MEYEIRLKPEEAEEWEIHETGHQ--TOFKVFDLYPGQKYLVOTRCK--PD
 GHR (172) LEYELQVKEVNETKWKMMDPILT--TSVPVYSLKVDKEYEVRVRSKQR-N
 cons (X) (W) (Y) R

G-CSFR (291) PGYWSPWSPGLQLRPTMKAPTIRLDTW
 RPLR (207) HGYWSRNSOESSVEWFNDPTLKDTTVW
 GHR (219) SGNYCEESEVLYVTLPQMSOFTCEEDE
 cons (G) W S W S

B

G-CSFR (376) LLFSEAQNVTLVAYNKAGTSS-PTTVVFLENEGPA-VTGLHAMAQDLN
 CONTAC (745) MPPSTQYQVRVKAFNSKGDGEFSLTAVIYSAQDAPTEVPTDVSVKVLSS

G-CSFR (422) TIWVDWEAPSLLP-QGYLTE-WEMSSPSYNNSYKSWMIEPNGNITGILLK
 CONTAC (795) EISVSWHRVTEKSVEGYQLRYWA---AHDKEAAQRVQVSNQEYSTRL

G-CSFR (470) DNINPFQLYRITVAPLYPGIVGPPVNVYTFAGERAPPHAEALHIKHV-GT
 CONTAC (840) ENLKKPNTRYNIDVSAPNSAGYGPPSRTIDIITRKAPPSQRRIISSVRSG

G-CSFR (519) TWAQLEWVPEAPRLGMIPLTHYTTFWADAGDHSFSVTLNISLHDFVLKHL
 CONTAC (890) SRYIITWDHVKAMSNESAVEGYKVLYRPDGQHE-GKLFSTGKHTIEW-PV

G-CSFR (569) EPASLYHVYLMATSRAGSTNSTGLTRTLDFSD
 CONTAC (938) PSDGEYVEVRAHNEGDGEVAQLKISGATACV

C

G-CSFR (602) LNIFTGILCLVLSTTCVVTWLCCKRRGKTSFWSDVPDPAHSSLSSWLPT
 IL-4R (209) LPLGVTISCLCLPLFCLFCDESITKIKKI-WWDQIETPARSPL---VA

G-CSFR (652) IMTEETFOLPSFWDSSVPSI
 IL-4R (253) IIIQDA-QVP-LWDKQTRSQ ---- 286 a.a.----

G-CSFR (672) TKITELEEDKKFTHWDSESSGNGSLFALVOAYVLQGDPREISNQSQPFSR
 IL-4R (557) VKQGAAQDPGVEGVRPSGDPGYKAFSSLLSSNGIRGDT--AAAGTDDGH

G-CSFR (722) TGDQVLYGQV-LESFISPGVMQY-TRSD-STQFLLGGPTPSFKSYENTWF
 IL-4R (604) GGYKPFQNPVPNQSPSSVPLFTFGLDTELSPSPLNSDPPKSPECLGLEL

G-CSFR (769) HSRPQETFVPQEPNQEDDCVFGPPFDFP-LFQGLQVHGVEE
 IL-4R (654) GLKGGDWVKAPEPADQVPKPFGDDLGFGIVYSSLTCHLCGH

D

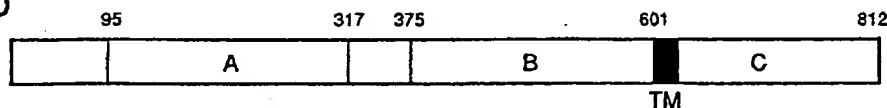


Figure 7. Comparison of the Amino Acid Sequence of the G-CSF Receptor with That of Other Growth Factor Receptors

(A) Alignment of the G-CSF receptor with prolactin and growth hormone receptors. The amino acid sequence from 95 to 317 of the murine G-CSF receptor is aligned with rat prolactin and human growth hormone receptors to give maximum homology by introducing several gaps (-). Identical residues in two sequences are enclosed by solid lines, and residues regarded as favored substitutions are enclosed by broken lines. Favored amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; F, Y, and W. Amino acids conserved in nine members of the growth factor receptor family (G-CSF, prolactin, growth hormone, erythropoietin, GM-CSF, IL-2B, IL-3, IL-4, and IL-6) are shown under each line with or without brackets. The residues without brackets are conserved in more than eight members, and the residues with brackets are conserved in five to seven members in the family.

(B) Alignment of the G-CSF receptor with contactin. The amino acid sequence from 376 to 601 of the mouse G-CSF receptor is aligned with the amino acid sequence of chicken contactin as described in (A).

polypeptide purified from NFS-60 cells is sufficient to bind G-CSF with a high affinity (R. F. E. I., and S. N., unpublished data) and little background binding of G-CSF to COS cells is observed.

Expression of the cloned cDNA gave rise to a protein that shows the same binding properties as those of the G-CSF receptor in mouse NFS-60 cells. Recombinant and native G-CSF receptors could bind 125 I-G-CSF with a dissociation constant of 200–300 pM (Figure 1), could specifically bind mouse G-CSF or human G-CSF, but could not bind other growth and differentiation factors such as IL-3, GM-CSF, M-CSF, IL-6, LIF, and prolactin (Figure 2). Furthermore, the cross-linking experiment indicated that the M_r of the recombinant G-CSF receptor (100,000–130,000) expressed in COS cells is similar to that of the G-CSF receptor purified from NFS-60 cells or the polypeptide cross-linked in NFS-60 cells (Figure 3). These results indicate that the G-CSF binding protein in NFS-60 cells is coded by the cloned cDNA. However, upon stimulation by G-CSF, it is not known whether this polypeptide alone can transduce the signal into cells or whether other proteins are required to form a functional receptor capable of conferring G-CSF responsiveness.

The amino acid sequence of murine G-CSF receptor (Figure 5) has many properties commonly found in the receptors for growth and differentiation factors (McDonald et al., 1989). It has a signal sequence at the N-terminal and a single transmembrane domain, suggesting that the N-terminal part of the molecule is extracellular and the C-terminal part remains inside the cell. Within the first 80 amino acids of the mature G-CSF receptor are four cysteines, two of which (Cys-21 and Cys-77) are organized in a manner characteristic of receptors of the immunoglobulin superfamily (Williams and Barclay, 1988). However, the sequence between these two cysteine residues does not seem to fulfill the criteria for recognition as a member of the immunoglobulin superfamily (Williams and Barclay, 1988).

Comparison of the amino acid sequence of the G-CSF receptor with all sequences in the National Biomedical Research Foundation data base revealed that one part of the extracellular domain of the G-CSF receptor has remarkable similarities with the prolactin receptor and another part has remarkable similarities with contactin (Figure 7). Prolactin is an anterior pituitary hormone and belongs to the family consisting of growth hormone, prolactin, and placental lactogen (Cooke et al., 1981). The amino acid sequences of human prolactin and growth hormone show a homology of 48% when conservative changes in amino acids are included. Accordingly, as shown in Figure 7A, the entire extracellular domain of the prolactin receptor (210 amino acids; Boutin et al., 1988) has a similarity of

43.2% (60 identical amino acids and 38 homologous amino acids) with a domain of the growth hormone receptor (Leung et al., 1987). To some extent, growth hormone competes with prolactin in binding to the prolactin receptor (Boutin et al., 1988) and vice versa (Leung et al., 1987). When the amino acid sequence from 96 to 317 of the mouse G-CSF receptor was aligned with the extracellular domain of the rat prolactin receptor, 54 of 227 amino acids were identical and 40 more represented conservative substitutions, yielding an overall similarity of 41.4%. The regions homologous between G-CSF and prolactin receptors are not well conserved in the growth hormone receptor, resulting in a low similarity (34.4%) between G-CSF and growth hormone receptors. Despite the similarity in amino acid sequence of the extracellular domains of the G-CSF and prolactin receptors, a 500-fold excess of unlabeled rat prolactin did not inhibit the binding of 125 I-G-CSF to the recombinant G-CSF receptor expressed in COS cells (Figure 2). This is consistent with the fact that the amino acid sequence of G-CSF has no significant homology to that of prolactin. These results may suggest that regions of the extracellular domain of the G-CSF receptor that are not similar to the prolactin receptor are required for the binding of G-CSF. In this regard, it is notable that the extracellular domain of the G-CSF receptor is 391 amino acids larger than that of the prolactin receptor.

When the sequences of the ligand binding domains of growth factor receptors were compiled, it was suggested that the receptors for growth hormone, prolactin, erythropoietin, and IL-6, as well as for the β chain of the IL-2 receptor, belong to a novel receptor family (Bazan, 1989). Recently isolated receptors for IL-4 (Mosley et al., 1989), IL-3 (Itoh et al., 1990), and GM-CSF (Gearing et al., 1989) are also members of this receptor family. The consensus amino acids in the family are indicated in Figure 7A. In the G-CSF receptor, the consensus cysteine and tryptophan residues are conserved, and the "WSXWS" motif (Gearing et al., 1989; Itoh et al., 1990) is also found at amino acid residues 294–298; this suggests that the G-CSF receptor belongs to the family. In this comparison of the G-CSF receptor with other hemopoietic growth factor receptors, it may be noteworthy that the similarity of the G-CSF and IL-6 receptors is less pronounced than that of the G-CSF and prolactin receptors, although G-CSF and IL-6 have a similarity of 44.6% (Nagata, 1990).

As shown in Figure 7B, the amino acid sequence from 376 to 601 in the extracellular domain of the G-CSF receptor has a significant similarity (42.9%) with a part of the extracellular domain of chicken contactin (Ranscht, 1988). Contactin is a neuronal cell surface glycoprotein of 130 kd and seems to be involved in cellular communication in the nervous system. Because the region from amino acid

(C) Alignment of the G-CSF receptor with the IL-4 receptor. The amino acid sequence from 602 to 808 of the mouse G-CSF receptor is aligned with two corresponding regions of mouse IL-4 receptor as above.

(D) Schematic representation of the mouse G-CSF receptor. The box indicates the mature G-CSF receptor. "TM" represents the transmembrane domain. Region "A" indicates a domain (222 amino acids) with similarity to other growth factor receptors, including prolactin and growth hormone receptors, and contains the "WSXWS" motif. Region "B" (226 amino acids) of the mouse G-CSF receptor shows similarity to chicken contactin. Region "C" (211 amino acids) includes the transmembrane domain (underlined) and the cytoplasmic domain of the G-CSF receptor and is similar to two regions of the mouse IL-4 receptor.

residues 737-818 of contactin can be aligned with the fibronectin type III segment involving binding to cells, heparin, and DNA, it is possible that this region plays an important role in cell adhesion (Ranscht, 1988). Granulopoiesis occurs daily in bone marrow, and the direct interaction of the neutrophilic progenitor cells with the bone marrow stroma cells has been proposed (Roberts et al., 1988). The similarity of part of the extracellular domain of the G-CSF receptor with contactin may suggest that this region is involved in the communication of neutrophilic progenitor cells and stroma cells.

The cytoplasmic domain consists of 187 amino acids and does not show any homology with the catalytic domain of the protein kinase family (Hanks et al., 1988). As observed in other growth factor receptors (Hatakeyama et al., 1989; Mosley et al., 1989), this region is rich in serine (12.8%) and proline (12.3%). When the transmembrane and cytoplasmic domains of the G-CSF receptor were aligned with the amino acid sequences of other growth factor receptors, a significant similarity with the IL-4 receptor was found. As shown in Figure 7C, the transmembrane domain and the first 46 amino acids of the cytoplasmic domain of the G-CSF receptor are homologous (50.0%) to the corresponding regions of the murine IL-4 receptor. Furthermore, amino acid residues 672-808 of the G-CSF receptor show significant similarity (45.4%) with amino acid residues 557-694 of the IL-4 receptor. These results suggest that signal transduction by G-CSF and IL-4 may be mediated by a similar mechanism.

The 3.7 kb mRNA for the G-CSF receptor was detected not only in NFS-60 cells but also in WEHI-3B D⁺ cells (Figure 6), suggesting that the same G-CSF receptor is involved in G-CSF-induced proliferation of NFS-60 cells and differentiation of WEHI-3B D⁺ cells. The different effects of G-CSF on NFS-60 and WEHI-3B D⁺ cells may therefore be mediated by different signal transduction mechanisms downstream of the receptor. In this regard, it is interesting that the *c-myc* and *evi-1* loci, which appear to be involved in differentiation of myeloid cells, are rearranged in NFS-60 cells but not in WEHI-3B D⁺ cells (Morishita et al., 1988). When RNAs from several mouse tissues were examined, the transcript for the G-CSF receptor was detected only in bone marrow cells that contain the progenitor for neutrophilic granulocytes. However, since G-CSF has some effect on bone remodeling (M. Y. Lee, R. F., T. J. Lee, J. L. Lottsfeldt, and S. N., submitted) and growth of endothelial cells (Bussolino et al., 1989), a low-level expression of the G-CSF receptor in other tissues cannot be ruled out. Under low-stringency hybridization, mRNA for the human G-CSF receptor could be detected in some human myeloid leukemia cells (R. F., Y. S., and S. N., unpublished data) using mouse G-CSF receptor cDNA as a probe. Availability of cDNA for the human G-CSF receptor would be valuable in the screening of various leukemia cells from human patients for the expression of the G-CSF receptor before treatment of the patients with G-CSF (Morstyn et al., 1989). Furthermore, the soluble form of the G-CSF receptor may be useful clinically to inhibit the proliferation of some human myeloid leukemia cells that are dependent upon G-CSF (Santoli et al., 1987).

Experimental Procedures

Cells

Mouse myeloid leukemia NFS-60 cells (Weinstein et al., 1986; kindly provided by J. Ihle, St. Jude Children's Research Hospital) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10-20 U/ml of recombinant mouse IL-3. COS-7 cells were routinely maintained in a Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS.

Recombinant Colony-Stimulating Factors

Human recombinant G-CSF was purified from medium conditioned with mouse C1271 cells, which were transformed with the bovine papillomavirus expression vector (Fukunaga et al., 1984) carrying human G-CSF cDNA (Tsuchiya et al., 1987). Mouse G-CSF was produced by using a similar expression system and purified to homogeneity (R. F., E. I., and S. N., unpublished data). Human recombinant G-CSF and M-CSF produced by Chinese hamster ovary cells were provided by Chugai Pharmaceutical Co. Human recombinant G-CSF produced by *E. coli* was purchased from Amersham. Mouse recombinant IL-3 and G-CSF were generous gifts from Dr. A. Miyajima and K. Arai (DNAX Institute). Mouse recombinant IL-6 and mouse recombinant LIF were generously provided by Dr. T. Hirano (Osaka University) and N. Nicola (Walter Eliza Hall Institute), respectively. Rat prolactin was purchased from Chemicon International, Inc.

Mouse recombinant G-CSF was radioiodinated by the IODO-GEN method (Fraker and Speck, 1978) with a slight modification (R. F., E. I., and S. N., unpublished data). Specific radioactivities ranged from $6-8 \times 10^4$ cpm/ng protein (1200-1600 cpm/fmol).

Construction of the CDM8 cDNA Library

Total RNA was prepared from exponentially growing NFS-60 cells by the guanidine isothiocyanate/CsCl method (Chirgwin et al., 1979), and poly(A) RNA was selected by oligo(dT)-cellulose column chromatography. Double-stranded cDNA was synthesized as described (Nagata et al., 1986a) using a kit from Amersham, except for the reverse transcriptase, which was purchased from Seikagaku Kogyo Co. Addition of BstXI nonpalindromic linkers to the blunt-end cDNA and size fractionation of cDNA on a 1% agarose gel were performed using a kit from Invitrogen. cDNA larger than 1.8 kb was recovered from the gel and ligated to BstXI-digested CDM8 vector (Seed, 1987). *E. coli* MC1061/p3 cells were transformed with the ligated DNA by the electroporation method as described (Dower et al., 1988).

DNA Preparation

A total of 6×10^4 bacterial colonies were plated on agar at a density of 60-80 colonies per well using 24-well microtiter plates, and glycerol cultures for each pool of colonies were prepared. LB broth was inoculated with aliquots from each glycerol culture, and plasmid DNAs were prepared by the boiling method (Maniatis et al., 1982) followed by phenol extraction and ethanol precipitation.

Transfection of COS-7 Cells

Monolayers of COS-7 cells were grown in 6-well microtiter plates, and transfection of plasmid DNA into COS-7 cells was carried out by a modification of the DEAE-dextran method (Sompayrac and Danna, 1981). In brief, about 50% confluent cells were washed three times with serum-free DMEM and incubated for 8 hr at 37°C with 0.6 ml of DMEM containing 50 mM Tris-HCl (pH 7.3), 0.3 mg/ml DEAE-dextran, and 1 µg of plasmid DNA. After glycerol shock with Tris-HCl-buffered saline containing 20% glycerol for 2 min at room temperature, cells were washed twice with DMEM and incubated in DMEM containing 10% FCS.

Screening of Transfectants of COS-7 Cells

At 72 hr after transfection, COS-7 cells were washed with DMEM containing 10% FCS and 20 mM HEPES (pH 7.3) (binding medium) and incubated at 37°C for 2 hr with 1.7×10^5 cpm (200 pM) of ¹²⁵I-G-CSF in 0.6 ml of the binding medium. Unbound radioiodinated G-CSF was removed, and cells were successively washed three times with phosphate-buffered saline (PBS) supplemented with 0.7 mM CaCl₂ and 0.5 mM MgCl₂ and once with PBS. Cells were then recovered by trypsinization, and the radioactivity associated with cells was counted using an ALTO-GAMMA 5000 MINAXI γ-counter (Packard). Background

binding of 125 I-G-CSF to COS-7 cells transfected with the CDM8 vector was 308 ± 38 (SD) cpm. Two positive pools were identified that showed significant binding of radiolabeled G-CSF (500 and 912 cpm) to the transfected COS-7 cells. Independent clones (144) from each positive pool were grown in six 24-well microtiter plates and subjected to sib selection (Maniatis et al., 1982) using a matrix of 12×12 clones. After a final round of miniprep preparation of plasmids and transfection into COS-7 cells, a single clone was identified from each positive pool.

Binding of 125 I-G-CSF to COS Cells and NFS-60 Cells

COS cells grown on 15 cm plates were transfected with 20 μ g of the p162 or pJ17 plasmid as described above except that cells were treated for 3 hr with a DEAE-dextran solution containing DNA. Cells were split into 6-well microtiter plates 12 hr after the glycerol shock and grown for 60 hr in DMEM containing 10% FCS. Cells were washed with binding medium and incubated at 4°C for 4 hr with 125 I-G-CSF (10 pM to 1.2 nM range) in 1.0 ml of the binding medium. To determine the non-specific binding of 125 I-G-CSF to cells, a large excess of unlabeled G-CSF (800 nM) was included in the assay mixture, and the radioactivity bound to the cells was subtracted from the total binding to yield the specific binding. For binding of G-CSF to NFS-60 cells, 5.2×10^6 cells were incubated at 4°C for 4 hr with various concentrations of 125 I-G-CSF in 0.3 ml of RPMI-1640 medium containing 10% FCS and 20 mM HEPES (pH 7.3).

Chemical Cross-Linking

The chemical cross-linking of 125 I-G-CSF to the receptor expressed in COS cells was performed according to the procedure described for NFS-60 cells (R. F., E. I., and S. N., unpublished data). In brief, 8×10^5 of COS cells (on 3.5 cm plate) transfected with the plasmid p162 were incubated at 4°C for 2.5 hr with 1.2 nM of the radiolabeled G-CSF in the presence or absence of 1.5 μ M unlabeled G-CSF in 0.6 ml of the binding medium. The cells were scraped from the plate using a cell lifter and washed with 1 ml of PBS three times. Cross-linking was carried out on ice for 20 min in 1 ml of PBS containing 150 μ M disuccinimidyl suberate (DSS) and 150 μ M disuccinimidyl tartarate (DST). The reaction was stopped by the addition of 50 μ l of 1 M Tris-HCl (pH 7.4), and cells were collected by centrifugation and lysed with 15 μ l of 1% Triton X-100 containing a mixture of protease inhibitors (2 mM EDTA, 2 mM (p-aminophenyl)methanesulfonyl fluoride hydrochloride, 2 mM O-phenanthroline, 0.1 mM leupeptin, 1 μ g/ml pepstatin A, and 100 U/ml aprotinin). After centrifugation, the clear lysate (10 μ l) was analyzed by electrophoresis on a 4%–20% gradient polyacrylamide gel in the presence of SDS (Laemmli, 1970).

Hybridization and Nucleotide Sequence Analysis

Colony hybridization and Northern hybridization were carried out as described (Maniatis et al., 1982). As a probe, the 2.5 kb HindIII-XbaI fragment of clone pJ17 was labeled with 32 P by the random primer labeling method (Feinberg and Vogelstein, 1983).

DNA sequencing was performed by the dideoxynucleotide chain termination method using T7-DNA polymerase (Pharmacia) and [α - 32 S]-dATPaS (Amersham).

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Expression Cloning of a Human Granulocyte Colony-stimulating Factor Receptor: A Structural Mosaic of Hematopoietin Receptor, Immunoglobulin, and Fibronectin Domains

By Alf Larsen, Terri Davis, Benson M. Curtis, Steve Gimpel, John E. Sims, David Cosman, Linda Park, Eric Sorensen, Carl J. March, and Craig A. Smith

From the Immunex Corporation, Seattle, Washington 98101

Summary

We report the isolation from a placental library, of two cDNAs that can encode high affinity receptors for granulocyte colony-stimulating factor (G-CSF) when expressed in COS-7 cells. The cDNAs are predicted to encode integral membrane proteins of 759 and 812 amino acids in length. The predicted extracellular and membrane spanning sequences of the two clones are identical, as are the first 96 amino acids of their respective cytoplasmic regions. Different COOH termini of 34 or 87 residues are predicted for the two cDNAs, due apparently to alternate splicing. The receptor with the longer cytoplasmic domain is the closest human homologue of the murine G-CSF receptor recently described by Fukunaga et al. (Fukunaga, R., E. Ishizaka-Ikeda, Y. Seto, and S. Nagata. 1990. *Cell* 61:341). A hybridization probe derived from the placental G-CSF receptor cDNA detects a ~3-kb transcript in RNAs isolated from placenta and a number of lymphoid and myeloid cells. The extracellular region of the G-CSF receptors is composed of four distinct types of structural domains, previously recognized in other cell surface proteins. In addition to the two domains of the HP receptor family-defining region (Patthy, L. 1990. *Cell* 61:13) it incorporates one NH₂-terminal Ig-like domain, and three additional repeats of fibronectin type III-like domains. The presence of both an NH₂-terminal Ig-like domain and multiple membrane-proximal FN3-like domains suggests that the G-CSF receptor may be derived from an ancestral NCAM-like molecule and that the G-CSF receptor may function in some adhesion or recognition events at the cell surface in addition to the binding of G-CSF.

Granulocyte colony-stimulating factor (G-CSF)¹ is a glycoprotein secreted by macrophages, fibroblasts, and endothelial cells originally identified by its ability to stimulate the survival, proliferation, and differentiation in vitro of predominantly neutrophilic granulocytes from bone marrow progenitors (1). The capacity of G-CSF to regulate in vivo granulopoiesis is supported by animal and clinical studies, which demonstrated a reversible rise in circulating neutrophil levels in response to administered recombinant G-CSF (2). G-CSF has pleiotropic effects on mature neutrophils, enhancing their survival and stimulating functional activation, including induction of neutrophil alkaline phosphatase (3) and high affinity IgA Fc receptors (4), priming for respiratory burst (5, 6), and increased chemotaxis (7). G-CSF effects

have also been observed on hematopoietic cells that are not committed to the granulocyte lineage, for example, stimulation of the proliferation or monocytic differentiation in vitro of some myeloid leukemic cells (8–10) and, in synergy with other colony-stimulating factors, the proliferation in vitro of some multipotential hematopoietic precursors (11–13). A recent clinical study implicates G-CSF in the regulation of human erythropoiesis (14). G-CSF may also affect nonhematopoietic cells, since it appears to stimulate the proliferation and migration of endothelial cells (15), and the growth of cell lines derived from colon adenocarcinomas (16) and small cell lung carcinomas (17).

These diverse effects of G-CSF are mediated by the interaction of G-CSF and specific cell surface receptors. Initial binding studies with native murine G-CSF detected low numbers of receptors on responsive murine cell lines and human bone marrow cells of the neutrophilic lineage (18–20). Affinity crosslinking studies suggested a murine receptor mo-

¹ Abbreviations used in this paper: FN3, fibronectin type III homology unit; G-CSF, granulocyte colony-stimulating factor; HP receptor, hematopoietin receptor; NCAM, neural cell adhesion molecule; PRL, prolactin.

molecular weight (M_r) of $\sim 150,000$ (21). G-CSF muteins with improved stability have been shown to bind a single class of sites ($K_d = 100\text{--}500$ pM) on circulating neutrophils (22), U937 cells (23), placental membranes, and trophoblasts (24). Similar affinities have been measured for the binding of native G-CSF to a single class of sites on myeloid leukemic and small cell lung carcinoma cell lines (17). Although affinity crosslinking experiments detected human receptors of $M_r \sim 150,000$ on neutrophils, an additional crosslink to a protein of $M_r \sim 120,000$ could be detected on placental membranes, suggesting a more complex receptor composition (22, 24).

Here we report the isolation from a placental library of two cDNA clones that encode high affinity receptors for G-CSF when expressed in COS-7 cells. The two clones encode identical extracellular and transmembrane sequences, but differ in the COOH-terminal portion of their cytoplasmic regions, due to what appears to be alternate splicing. The predicted protein sequence of one clone suggests it is the strict human homologue of a recently cloned murine G-CSF receptor cDNA (25).

Materials and Methods

Human G-CSF Preparation. Human G-CSF (26) was expressed in yeast, using the α -factor secretion system (27), as a mutein in which Cys17 was replaced by serine and Arg22, by lysine. These alterations inhibit, respectively, the formation of disulfide-linked oligomers and inappropriate processing through destruction of a KexII recognition site. Alternatively, a fusion polypeptide of the same construct but incorporating a hydrophilic octapeptide at the NH₂ terminus to aid in purification (28) was also expressed. The biological activities of both purified forms were $\sim 2 \times 10^7$ U/mg determined in a standard proliferation assay using the murine myeloid leukemic cell line DA-1. 1 U corresponds to that amount of G-CSF that gives half-maximal [³H]TdR incorporation.

Radiolabeling of G-CSF. Purified human G-CSF was radiolabeled to a specific activity of 7×10^{14} cpm/mmol using a solid-phase chloramine-T analogue. 5 μ g of purified G-CSF and 2 μ Ci Na¹²⁵I in 150 μ l PBS was placed in a 10 \times 75 mm glass tube previously coated with 5 μ g of Iodogen (Pierce Chemical Co., Rockford, IL) and incubated for 25 min, 4°C. Free and ligand-bound iodine were subsequently separated by gel filtration through a 1 ml column of Biogel P-6 (BioRad Laboratories, Richmond, CA) that had been blocked with BSA. Radiolabeled stocks were stored at 4°C in RPMI-1640 containing 2.5% BSA, 20 mM Hepes buffer, and 0.2% sodium azide, pH 7.2 (binding medium). The specific radioactivities of labeled G-CSF were based on determination of initial protein concentration by amino acid analysis, with correction from control experiments to determine protein recovery after iodination, in which an aliquot of G-CSF was mixed with ¹²⁵I-G-CSF and the iodination protocol repeated, with omission of Na¹²⁵I.

Cell Lines and Tissue Preparations. HL60, U937, C10, KG-1, HeLa, RAJI, MJ, and RPMI 1788 cell lines were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY), 10% FCS, 2 mM glutamine, and 50 μ M 2-ME at 37°C in a humidified atmosphere of 5% CO₂ in air. Peripheral blood granulocytes were isolated by sedimentation on discontinuous Ficoll Hypaque (Sigma Chemical Co., St. Louis, MO) gradients followed by red blood cell lysis in ammonium chloride. Placental membranes were isolated as follows. Fresh, full-term human placenta, obtained from Swedish

Hospital Medical Center (Seattle, WA) on ice, were washed six times with ice-cold PBS to extract excess blood, and the tissue, trimmed of amnion and chorion, was cut into small pieces. The pieces were transferred to one volume of Buffer A (30 mM Hepes, pH 7.4, containing 0.25 M sucrose, 1 mM PMSF, 1 μ M pepstatin A, 10 μ M leupeptin, 2 mM *o*-phenanthroline, and 0.02 U/ml aprotinin), homogenized for five 1-min intervals with a PT10/35 homogenizer (Brinkman, Westbury, NY) at setting 7 and centrifuged at 6,800 *g* for 30 min, 4°C. The supernatant was made 0.1 M in sodium chloride and 0.2 mM in magnesium chloride and centrifuged at 42,000 *g* for 40 min at 4°C. The resulting pellets were washed twice in Buffer A by resuspension and centrifugation as above. The final sedimented membranes were resuspended in 30 ml of Buffer A at a protein concentration of 10–20 mg/ml and stored at -70°C .

Binding Assays and Data Analysis. For equilibrium binding assays with native (placental membrane) G-CSF receptor, serial dilutions of ¹²⁵I-G-CSF in binding media were incubated with 300 μ g membrane (protein) in 10 \times 75 mm glass tubes in a total volume of 100 μ l for 2 h, 4°C. Control experiments showed equilibration had been reached in this time. Bound ligand was measured by subsequent collection of membranes in the reaction mixture on glass microfiber filters (Whatman, Hillsboro, OR) using a vacuum filtration apparatus. Filters were washed three times with ice-cold PBS/BSA (1 mg/ml) before gamma counting. Nonspecific binding was determined for each data point with a control tube containing a 400-fold molar excess of unlabeled G-CSF. Free radiolabeled ligand for each data point was measured by counting an aliquot of ligand identically incubated in the absence of membranes, after subtraction of the corresponding bound counts. Binding curves were plotted in the Scatchard coordinate system, expressing bound ligand in units of fmole/milligram membrane protein.

For equilibrium binding assays with recombinant G-CSF receptor, COS-7 cells transfected with either the D-7 or 25-1 G-CSF receptor cDNA clone (COS-G-CSFr) were first diluted 10-fold with carrier cells (EL4-3⁺ murine T cells) to prevent COS cell aggregation. EL4-3⁺ and untransfected COS cells were both shown to lack receptors for human G-CSF. Serial dilutions of ¹²⁵I-G-CSF in binding media were incubated with cells (2×10^6 total cells/ml) for 2 h at 4°C in a total volume of 150 μ l using 96-well microtiter plates. Free and bound ligand were separated by centrifugation of duplicate 60- μ l aliquots of the reaction mixture in plastic tubes containing a phthalate oil mixture (29). The tubes were cut, and supernatant (free ligand) and pellets (bound ligand) were gamma counted. Nonspecific binding was determined by inclusion of a 200-fold molar excess of unlabeled G-CSF in the reaction mixture at one ligand dilution; the linearly extrapolated nonspecific binding was subtracted from each data point to generate specific binding. Binding parameters determined on adherent COS-G-CSF receptor cells were similar to those determined in the suspension assay.

Affinity Cross-linking. Adherent COS cells on 10-cm culture dishes transiently expressing the recombinant G-CSF receptor were incubated with ¹²⁵I-G-CSF (1 nM) in RPMI 1640 for 2 h at 4°C in the presence or absence of unlabeled G-CSF (1 μ M). Cells were washed twice in ice-cold PBS and then crosslinked in situ with 0.1 mg/ml bis-(sulfosuccinimidyl) suberate (BS³, Pierce Chemical Co.) in PBS at 25°C for 30 min. Cells were subsequently washed twice with PBS and then lysed with 0.5 ml of PBS/1% Triton containing protease inhibitors (2 mM PMSF, 10 μ M pepstatin A, 10 μ M leupeptin, 2 mM *o*-phenanthroline, 2 mM EGTA, 1.25 mM benzamidine, 0.5 mM EDTA, and 2 μ g/ml soybean trypsin inhibitor). Lysates were scraped from plates, microfuged at 12,000 *g* for 10 min, and supernatants retained. Placental membranes (8 mg pro-

tein/ml) were incubated in 1.5-ml plastic microfuge tubes with 1 mM ^{125}I -G-CSF in a total volume of 100 μl PBS for 2 h at 4°C in the presence or absence of unlabeled G-CSF (1 μM). Membranes were then washed two times with ice cold PBS, resuspended in 100 μl of PBS, and incubated with BS³ (0.1 mg/ml) for 30 min at 25°C. Membranes were washed twice, then lysed in 150 μl of PBS 1% Triton (with protease inhibitors) for 30 min at 4°C. Insoluble debris was removed by centrifugation for 30 min at 10,000 g, and the supernatant was retained.

SDS-PAGE. Samples, including methyl ^{14}C -labeled molecular weight markers (Bethesda Research Laboratories, Bethesda, MD), were boiled for 30 min in sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME) and analyzed on an 8% SDS gel (30). After electrophoresis, gels were fixed in 25% isopropanol, 10% acetic acid, dried, and autoradiographed with Kodak X-Omat AR film at -70°C.

cDNA Library Construction and Screening. Total cell RNA was isolated from whole fresh placental tissue as described below and polyadenylated RNA prepared by chromatography on oligo(dT)-cellulose as described (31). Double-stranded, oligo(dT)-primed cDNA was prepared with a commercial kit (Amersham Corp., Arlington Heights, IL). The resulting cDNA was size fractionated by chromatography on Sephacryl S-1000 (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.5 M sodium acetate. The excluded cDNA was cloned into the BglIII site of the mammalian expression vector, pDC302 (32) by an adaptor method similar to that described by Haymerle et al. (33). Briefly, noncomplementary oligonucleotides of the sequence 5'-GATCTTGGAACGAGACGACCTGCT and 5'-AGCAGGTCGTCGTTCCAA synthesized on a DNA synthesizer (model 380A; Applied Biosystems, Foster City, CA) were annealed and ligated in separate reactions to either cDNA or BglIII cut vector. Nonligated oligonucleotides were separated from cDNA or vector by chromatography over Sepharose CL-2B (Pharmacia Fine Chemicals) at 65°C in 10 mM Tris (pH 8.0), 0.1 mM EDTA. 5 ng of adaptor vector was ligated to adaptor cDNA in 10- μl reactions containing 50 mM sodium chloride, 50 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 1 mM spermidine, 0.5 mM ATP, 0.1 U/ μl T4 polynucleotide kinase and 0.4 U/ μl T4 DNA ligase for 30 min at 37°C. Reactions were then desalted by drop dialysis on VSWP 013 filters (Millipore Corp., Bedford, MA) against distilled water for 40 min immediately before electroporation into *Escherichia coli* strain DH5 α as described (34). Transformants were obtained with an average cDNA insert size of 1.6 kb. Pools of 600 colonies were prepared and DNA minipreps of these were transfected into COS cells as described (35). After 3 d growth in DME/10% FCS, the cells were screened for ^{125}I -G-CSF binding by an in situ autoradiographic plate binding assay (36).

RNA Analysis. Total cellular RNAs were isolated by the guanidinium isothiocyanate-caesium chloride method and electrophoresed through formaldehyde agarose gels as described (31). RNA was transferred to nylon filters (Amersham) by capillary blotting and UV crosslinked using a Stratalinker (Stratagene, La Jolla, CA). Filters were probed with a ^{32}P -labeled antisense RNA prepared by T7 RNA polymerase transcription of a subclone of the D7 cDNA in pBlueSK (Stratagene). High stringency blot hybridization and washing conditions were as previously described (35).

Sequence Analysis. Sequences were aligned using various computer programs (GAP; 37, 38) and the progressive alignment method of Feng and Doolittle (39) as well as by visual inspection. With the exception of the alignment between the human and murine G-CSF receptors, a consensus alignment was generated for all sequences, rather than optimizing the alignment between any given pair of sequences. Alignment scores were generated using the NBRF

program ALIGN using the MD data matrix with a bias of +6 and a gap penalty of 6. The prediction of residues involved in β -strands in immunoglobulin domain folding patterns used the turn and secondary structure prediction algorithms of Cohen et al. (40), the hydrophobic moment algorithm of Eisenberg et al. (41) as well as by inspection.

Results

Isolation of Human G-CSF Receptor cDNAs. Quantitative binding studies using radioiodinated G-CSF on a panel of human cell lines demonstrated low level expression of a single class of binding sites ($N < 1,000/\text{cell}$, $K_d \sim 1 \text{ nM}$; data not shown). G-CSF receptors with a similar affinity were detected on placental membranes (see below), but at a level of $\sim 200 \text{ fmol/mg}$. Since binding of epidermal growth factor to A431 cell membranes at this level would correspond to a site number of $\sim 10^6/\text{cell}$ (42) we concluded that G-CSF receptors were expressed at unusually high levels in placental tissue. A placental cDNA library was prepared in a mammalian expression vector and DNA from pools of ~ 600 transformants were transfected into COS cell that were then screened for ^{125}I -G-CSF binding by contact autoradiography (37). A positive clone, D7, was obtained after screening 20 pools and contained a 2.6-kb cDNA insert that was used as a hybridization probe to identify three additional related clones from the same library. Restriction digests and DNA sequencing showed that the cDNA clones fell into two classes: three were of the D7 type and one of a somewhat different form, 25-1, shown in Fig. 1. The 25-1 clone differs from the D7 clones only in its lack of a poly(A) tract and in the presence of a 419-bp internal sequence insert. This insert occurs between nt 2411 and 2412 of the D7 cDNA and appears to be derived from an unspliced intron since it contains splice donor and acceptor consensus sequences at the junctions with the D7 sequence.

DNA sequencing of these clones showed that the first ATG occurs in a context corresponding well to the Kozak consensus sequence (CCA/GCCATG; 43) and initiates a reading frame that terminates after 11 codons. The next potential initiation codon occurs 45 nt downstream, within an inferior Kozak context. This reading frame encodes proteins of 783 and 836 amino acids in the D7 and 25-1 cDNAs, respectively. Hydropathy analysis identified two major hydrophobic regions in the sequence (Fig. 1 D). The first, at the NH₂ terminus, is a presumed hydrophobic signal sequence of 24 residues; the second, between residues 604 and 629, is a presumed transmembrane domain that makes a single helical span (Fig. 1 B). Both forms of receptor are thus composed of an extracellular region of 603 amino acids and a transmembrane region of 26 amino acids, but differ in the predicted COOH-terminal portions of their cytoplasmic domains. The protein encoded by cDNA D7 has a cytoplasmic domain of 130 amino acids, while the unspliced intron sequence inserted in clone 25-1 after amino acid 725 predicts a cytoplasmic domain of 183 residues. The COOH-terminal amino acid sequence of D7 appears significantly more hydrophobic than that of 25-1 and contains one less Cys residue (Fig. 1). Both the D7 and 25-1 cytoplasmic sequences have high contents of proline (14.6 and



Figure 1. Human G-CSF receptor cDNAs. (A) Schematic representation and restriction map of G-CSF receptor cDNA clones D7 and 25-1. Restriction sites are indicated for BamHI (B) and SstI (S). The solid arrow marks the position in the D7 sequence at which the 25-1-specific insertion occurs. The insert sequence present only in the 25-1 clone is indicated as a filled bar, all other sequence is identical in the two clones. The deduced coding sequences are shown as 25 wide bars, noncoding sequences as narrow bars. The predicted signal transfer and transmembrane sequences are shown crosshatched. The 35-residue poly(A) tail of the D7 clone is shown as A35. (B) The nucleotide and deduced amino acid sequence of clone D7. The signal peptide cleavage site predicted by the probability weight matrix of von Heijne (75) is shown by an open arrow, and the predicted NH₂ terminus of the mature protein is designated residue 1. The predicted membrane spanning sequence is indicated by heavy underline and the Trp-Ser motif by a light underline. Cysteine residues are boxed and potential N-linked glycosylation sites are indicated by asterisks. The position of the 25-1 intron insertion point is indicated by a solid arrow. (C) The nucleotide and deduced amino acid sequence of the 3' end of clone 25-1. Enumeration is continuous with B. Cysteine residues are boxed. Solid arrows indicate the junctions of the 419-bp insert with sequences common to both 25-1 and D7. (D) On facing page Hydropathicity plot of the D7 and 25-1 receptor sequence according to the method of Kyte and Doolittle (76). The predicted signal and membrane-spanning sequences are indicated by arrows. These sequence data are available from EMBL/Genbank/DDJB under the accession numbers X55720 (clone D7) and X55721 (clone 25-1).

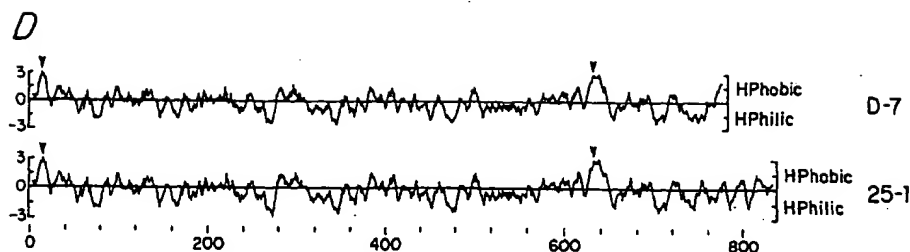
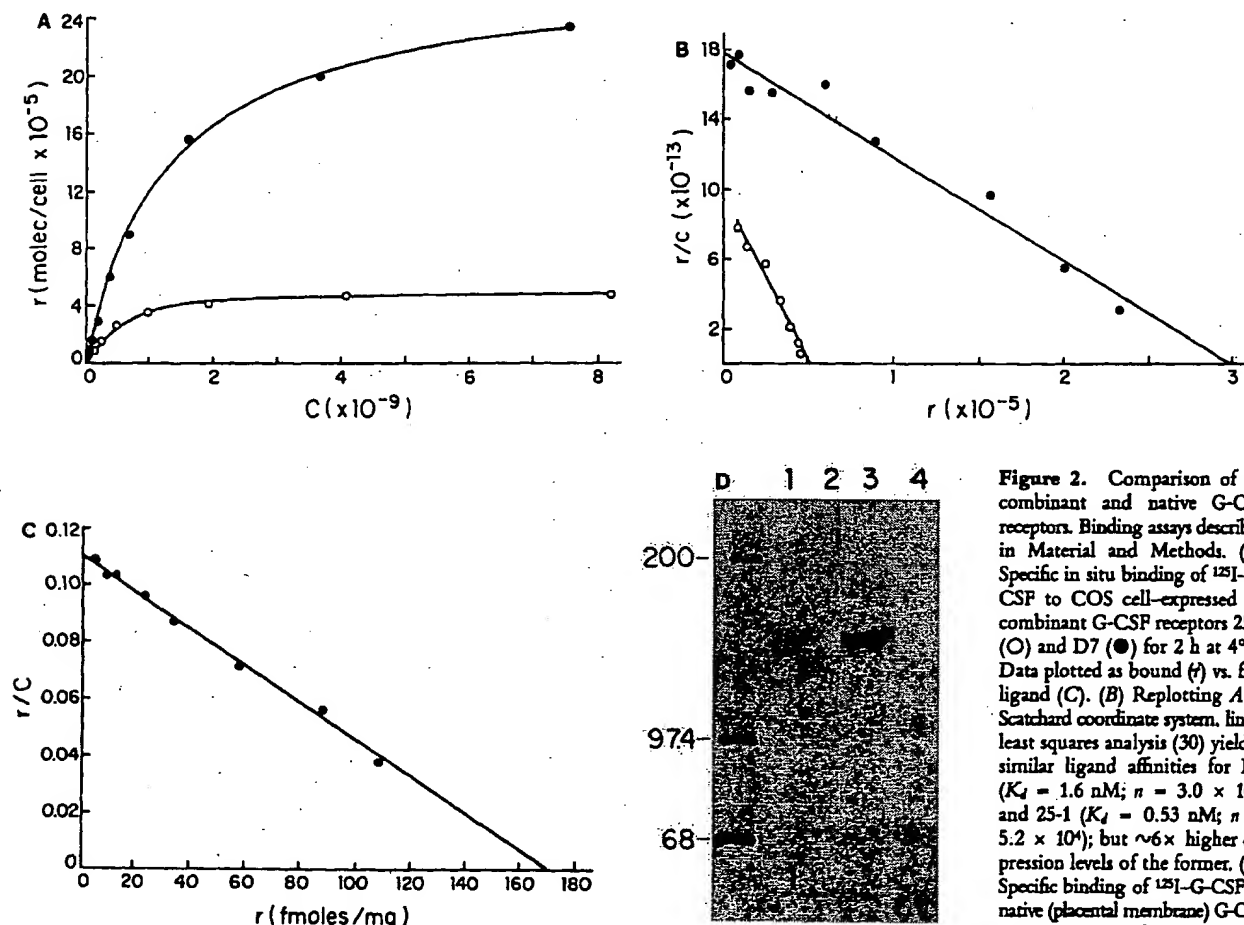


Figure 1 D.

13.1%, respectively) and serine (13 and 10.4%) a property noted for the cytoplasmic domains of many members of the hematopoietin (HP) receptor family (44). Neither the D7 nor 25-1 cytoplasmic regions contain sequences indicative of tyrosine kinase activity (45) but Ser760 of the 25-1 receptor represents a potential protein kinase C phosphorylation site (46). The predicted sequences of both G-CSF receptors con-

tain nine potential N-linked glycosylation sites (Fig. 1 B), all but one in the proposed extracellular region. A murine G-CSF receptor cDNA isolated from myeloid leukemia cell library (25) encodes a predicted mature protein of 812 amino acids, identical in length to that of 25-1, and its COOH-terminal sequence is homologous to that of 25-1 but to D7 only up to the position of the proposed splice site. The 25-1



0.70 nM, similar to both recombinant receptors. (D) Affinity cross-linking of native (placental; lanes 1 and 2) and recombinant (COS-expressed; lanes 3 and 4) in the absence (1 and 3) or presence (lanes 2 and 4) of a 200-fold molar excess of unlabeled G-CSF. The calculated receptor M_r , after subtraction of ligand molecular weight, is $\sim 150,000$ in both cases. Cross-linking conditions described in Materials and Methods.

Figure 2. Comparison of recombinant and native G-CSF receptors. Binding assays described in Material and Methods. (A) Specific in situ binding of ^{125}I -G-CSF to COS cell-expressed recombinant G-CSF receptors 25-1 (O) and D7 (●) for 2 h at 4°C. Data plotted as bound (r) vs. free ligand (C). (B) Replotting A in Scatchard coordinate system. linear least squares analysis (30) yielded similar ligand affinities for D7 ($K_d = 1.6$ nM; $n = 3.0 \times 10^3$) and 25-1 ($K_d = 0.53$ nM; $n = 5.2 \times 10^4$); but $\sim 6\times$ higher expression levels of the former. (C) Specific binding of ^{125}I -G-CSF to native (placental membrane) G-CSF receptors. Linear least squares fit to Scatchard plot give a K_d of

cDNA appears, therefore, to encode the strict human homologue of the murine receptor. At the protein level, these homologues are highly conserved in sequence (62% identity).

Comparison of Native and Recombinant G-CSF Receptors. The ligand-binding and affinity cross-linking characteristics of the recombinant G-CSF receptors encoded by D7 and 25-1, expressed in COS cells, are compared with those of native (placental) receptors in Fig. 2. All three receptors display a single class of binding sites, with equilibrium dissociation constants of 1.6 nM (D7), 0.53 nM (25-1), and 0.67 nM (placental). Expression levels of the D7 clone, however, were approximately sixfold higher than 25-1 (298,000 v. 52,000 sites/cell). Affinity cross-linking studies of COS-expressed D7 and placental G-CSF receptors detected a single subunit in each case with an apparent M_r of $\sim 150,000$. As the calculated protein molecular mass of the recombinant receptors are 92 kD (25-1) and 86 kD (D7), the G-CSF receptor is estimated to contain $\sim 35\%$ carbohydrate by weight. Thus both native and recombinant receptors share similar characteristics.

Expression of G-CSF Receptor mRNA. An antisense RNA transcript of the entire D7 sequence was used to probe Northern blots of total cellular RNAs isolated from a variety of sources (Fig. 3). A hybridizing band of ~ 3 kb was detected in placental RNA samples (lane 4) and RNA isolated from human hematopoietic cells previously reported to express G-CSF receptors (17, 22, 23, 47), including the myelogenous leukemia cell line KG-1 (Fig. 3, lane 7), the promyelocytic cell line HL-60 (lane 1), the premonocytic cell line U937 (lane 5), bone marrow cells (lane 7), and peripheral blood granulocytes (lanes 2, 3), the latter containing particularly high levels, consistent with the prominent G-CSF responsiveness of this cell type. The KG-1 and peripheral blood granulocyte samples both show a minor additional hybridizing species at ~ 7 kb (lanes 2, 3, and 7), as did placental RNA upon longer exposure (data not shown). This species was not detectable in cytoplasmic placental RNA, suggesting it is a nuclear precursor (data not shown). The observed pattern of expression suggests that one or both of the G-CSF receptors cloned from the placental library also encode the receptors used by hematopoietic cells. Unexpectedly, we have also detected low levels of these transcripts in the HTLV-1-transformed T cell lines C-10 and MJ (lanes 6, 10) and the B lymphoblastoid cell lines RAJI and RPMI 1788 (lanes 9, 11), cells that are of lymphoid not myeloid lineage. The significance of this observation is unclear and requires further investigation. Under the stringent hybridization conditions used, no specific hybridization was seen with total RNAs isolated from HeLa cells (lane 8), dermal fibroblasts, brain, or COS cells (data not shown).

Domain Structure and Sequence Homology of the G-CSF Receptor. A computer search of several databases queried with the entire G-CSF receptor sequence revealed significant homology of the G-CSF receptor extracellular region to three distinct groups of sequences: (a) members of the Ig superfamily (48), (b) the extracellular regions of all members of the recently identified hematopoietin (HP) receptor family

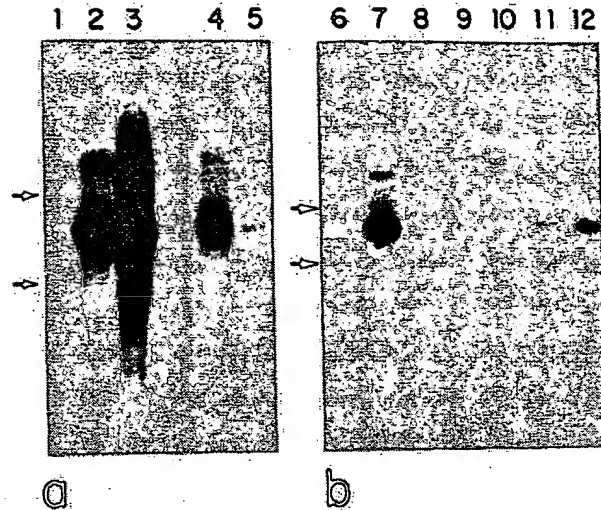


Figure 3. G-CSF receptor RNA analysis. Northern blots of human total cellular RNAs electrophoresed on formaldehyde agarose gels, hybridized with antisense D7 probe, and washed as described in Materials and Methods. The positions of the 18S and 28S ribosomal RNAs are indicated by arrows. All lanes contain 2.5 μ g of total cellular RNA except lane 3, which has 0.5 μ g of total RNA to avoid overexposure. The blots were exposed at minus 80° for different times. (A) 2-h exposure of a blot of RNA samples from the following sources: HL-60 (lane 1), peripheral blood granulocytes (lanes 2, 3), placenta (lane 4), U937 (lane 5). (B) 24-h exposure of a blot of RNAs isolated from the following sources: C-10 (lane 6), KG-1 (lane 7), HeLa (lane 8), RAJI (lane 9), MJ (lane 10), RPMI 1788 (lane 11), aspirated pelvic bone marrow (lane 12).

(44, 49–53) and (c) the type III homology units (FN3) of several vertebrate fibronectins (54) and neural cell adhesion molecules (NCAMs) (55, 56). Each of these homologies is localized to discrete regions of the extracellular portion of the G-CSF receptor. The NH₂-terminal 90 residues of the G-CSF receptor show statistically significant alignment scores (>3 SD) with several members of the Ig superfamily (data not shown). Fig. 4 A shows a consensus alignment of these NH₂-terminal residues with the NH₂-terminal sequences from the murine G-CSF receptor (25), human IL-6 receptor (57), and light chain domains of Ig NEW (58, 59). The G-CSF receptor contains an invariant Trp, two appropriately spaced Cys, and other residues in conserved positions that define Ig-like domains (48). Cys23 and Cys79 of the G-CSF receptor, therefore, are likely to form a disulfide loop characteristic of the Ig fold (60). This structural motif is further supported by the presence of sequences predicted to form the β strands characteristic of Ig domains (underlined Fig. 4 A).

The ~ 200 residue segment of the G-CSF receptor following the Ig-like moiety (Arg94 to Pro 299) shows strong homology to extracellular regions of all members of the HP receptor family (ALIGN scores Fig. 4 D) (44) and is shown aligned with the corresponding segments of the murine receptor and other selected family members in Fig. 4, B and C. This region contains the sequence features that define the

HP receptor family; notably a Trp and four Cys residues (boxes, asterisks: Fig. 4 B) conforming to a conserved pattern (49–53) found in nearly all HP receptors (44) and a COOH-terminal WSXWS motif (50–53) which has proven to be a hallmark of the HP receptor family (44). The four Cys residues form two successive disulfide loops in the growth hormone receptor (61), and it is likely that a similar pairing occurs in the G-CSF receptor between Cys107 and 118, and Cys153 and 162. A recent sequence analysis of the HP receptor family-defining region (62) has suggested it can be resolved into two distinct elements each ~100 residues in length: one NH₂-terminal “cysteine-rich” region and a COOH-terminal “cysteine-poor” region, the latter homologous to fibronectin type III repeats. Although in the G-CSF receptor these two regions do not differ significantly in number of Cys residues (NH₂-terminal, 6; COOH-terminal, 5), the last 100 residues of the region do contain the sequence features characteristic of FN3 repeats, as shown by alignment with examples of FN3 repeats from human fibronectin (54) and two neural cell adhesion molecules, murine L1 (55) and *Drosophila* neuroglian (63) in Fig. 4 C. The FN3-like character of the region is demonstrated by the presence of three residues, Trp229, Leu274, and Tyr279 (asterisks), in the pattern which is the sole sequence feature absolutely conserved in all of the type III repeats of fibronectin (54, 64). Although this region of the G-CSF receptor exceeds the low Trp and Cys content characteristic of FN3 repeats (54) its designation as an FN3-like domain is further supported by statistically significant ALIGN scores when compared with 14 of the 16 type III repeats of human fibronectin (data not shown).

The ~300 residues of the G-CSF receptor bordered by the WSXWS motif and the transmembrane region also show significant homology to FN3 repeats of several vertebrate fibronectins and NCAMs, suggesting this region consists of three additional repeats of this element. These proposed FN3-like domains are shown aligned with the corresponding segments of the murine receptor and the examples of FN3 repeats in Fig. 4 C. These three FN3-like domains of the G-CSF receptor contain the conserved Trp and Tyr residues, described above, but only the last domain contains the hallmark Leu, a residue only partially conserved in NCAM FN3 repeats. These domains of the G-CSF receptor also contain a pair of aromatic residues common to the FN3-like domains of many NCAMs and fibronectins (boxed). The four proposed FN3-like domains of the G-CSF receptor each contain at least three Trp residues, and in this respect resemble the FN3 repeats of NCAMs rather than those of fibronectin. The proposed second, third, and fourth FN3 domains of the G-CSF receptor gave significant ALIGN scores respectively to 4, 13, and 15, of the 16 type III repeats of human fibronectin (data not shown).

Discussion

Here we report the isolation and characterization of two distinct types of G-CSF receptor cDNAs from a human placental library. The equilibrium ligand binding and cross-linking characteristics of the recombinant receptors are similar

to those of native receptors on placental membranes (Fig. 2). The D7 and 25-1 cDNAs are predicted to encode integral membrane glycoproteins, 759 and 812 amino acids in mature length, respectively. The predicted molecular masses of these receptors, 86 and 92 kD, are substantially less than the ~150 kD inferred by affinity crosslinking, suggesting that some or all of the nine potential N-linked glycosylation sites contain carbohydrate. The two receptors share identical extracellular (603 aa) and transmembrane (26 aa) regions, as well as the first 96 residues in their cytoplasmic regions, but have alternate COOH-terminal sequences of 34 residues (D7) and 87 residues (25-1). The nucleotide sequences of the two cDNAs indicate that they are probably derived from alternatively processed transcripts of the same gene, since they differ only with respect to a 419-bp insert in the 25-1 cDNA that appears to be an unspliced intron. A recently reported murine G-CSF receptor cDNA (25) encodes a protein that shows strong homology to both placental G-CSF receptors up to the splice point in the cytoplasmic domain, after which the homology continues only in the 25-1 clone, indicating it is the strict human homologue of the reported murine receptor. It is unclear if the reported murine G-CSF receptor cDNA sequence has the splicing potential to encode an alternate COOH-terminus. Inspection of the murine sequence reveals a very similar (murine, CAG GTCCTC; human, CAG GTCCTT) potential splice donor sequence located within the same DQ/VLY peptide sequence as the human 25-1 cDNA. However, while there are potential splice acceptor sites in the murine sequence, translation of the sequences downstream in all three reading frames reveal no significant homology to the D7 type COOH terminus. Thus, generation of a D7-type cytoplasmic terminus in murine G-CSF receptors might be possible if an alternative splice acceptor site exists in 3' sequences of the murine gene.

Differential splicing results in the tissue-specific expression of transcripts encoding alternate cytoplasmic domains for at least two other cell surface proteins, rat liver prolactin (PRL) receptor (65) and chicken NCAM (66). The tissue specificity of expression of the potential G-CSF receptor isoforms remains to be determined at both the mRNA and protein level, but a preliminary analysis with specific oligonucleotide probes suggests human granulocytes express predominantly transcripts of the 25-1 type (data not shown). This raises the possibility that the D7 receptor is specifically expressed at higher levels in nonhematopoietic cells such as placenta, and suggests that the alternate cytoplasmic domains may confer functional differences to the two receptors. It has been proposed that the smaller form of the PRL receptor functions in ligand transport across epithelial barriers in liver rather than in signal transduction (65). By analogy the D7 isoform of the G-CSF receptor may serve to transport or sequester G-CSF in placental tissues. Alternatively, the two isoforms may differ in signal transduction properties, reflecting in part, the diverse biological effects of G-CSF. While the signal transduction mechanism of the G-CSF receptor is unclear, it is interesting that the 25-1 receptor, unlike D7, does contain one potential C kinase phosphorylation site (46). Signal transduction may also be effected through a distinct subunit with which the ligand-

G-CSF receptor complex interacts, as is found in the gp130-IL-6 receptor system (67). Conceivably, this subunit may be gp130 itself, and it is interesting, in this regard, that IL-6 and G-CSF show significant sequence homology (68), and their receptors share a similar domain composition (see below). Both G-CSF receptor cytoplasmic domains contain a high proportion of Pro and Ser residues, like those of many other HP receptor family members (44), the significance of which remains to be elucidated. The cytoplasmic sequence of these receptors may influence stability, cellular localization, or association with other membrane proteins.

The extracellular region of the G-CSF receptor consists of three distinct regions of homology to other cell surface proteins: (a) a ~90 residue NH₂-terminal Ig-like region, (b) a ~200 residue HP receptor superfamily-defining region, and (c) ~300 residues of three tandem FN3-like repeats. Consistent with the proposal of Pathy (62), the COOH-terminal 100 residue segment of the HP receptor family-defining region of the G-CSF receptor appears to be an FN3-like domain, albeit one with an elevated Trp and Cys content that is unique among FN3 repeats. Since many protein domains are ~100 residues in length, it is likely that the NH₂-terminal "cysteine-rich" or double-loop region of the HP receptor-defining region is, like the WSXWS-containing FN3 element, a discrete structural domain. Thus, the structure of the G-CSF receptor extracellular region can be resolved into the 6 domains of ~100 residues each shown schematically in Fig. 5. By this analysis, the G-CSF receptor appears to be a mosaic of four types of domains, two found associated only in HP receptors (double-loop and WSXWS-FN3) and two found together in NCAMs (NH₂-terminal Ig and membrane-proximal FN3). Given the likelihood that the Ig superfamily molecules of the immune system arose from NCAM-like ancestors (48), it is tempting to speculate that the G-CSF receptor retains the domain structure of an early intermediate in the evolution of the HP receptor superfamily from these same ancestors. Successive deletion of the terminal extracellular domains of a G-CSF receptor-like molecule could thus yield all known HP receptor domain structures; deletion of the three FN3 domains yields an IL-6 receptor-like structure, and further deletion of the Ig domain leads to the core domain structure of most HP receptors, which is duplicated in the case of the IL-3 receptor. Key to such a proposal is determination of the origin of the cysteine-rich or double-loop domain, thus far a unique domain feature found only in the HP receptors, but which, like the Ig and FN3 domains, may also have arisen from a domain in some NCAM-like ancestor.

The function of these structural domains in the G-CSF receptor is unclear. The HP receptor family-defining region, which comprises the entire extracellular region of many of

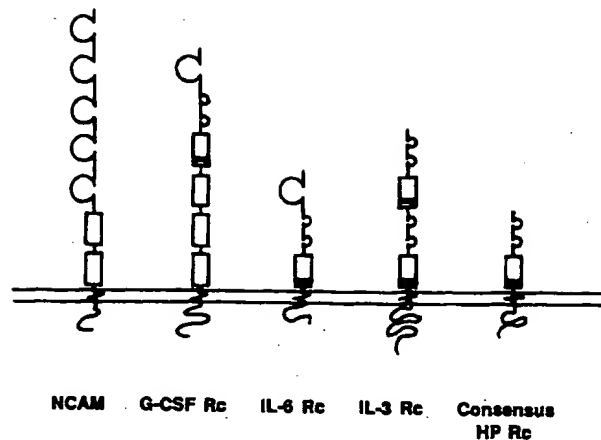


Figure 5. Schematic representation of the nominal structure of hematopoietin receptors and a typical neural cell adhesion molecule. Ig-like domains are shown as large loops. FN3 domains are shown as rectangular boxes. The cysteine-rich, double-loop domains of HP receptor family-defining regions are shown as a pair of small loops, to represent the disulfide pairing determined for human growth hormone receptor (61). The FN3-like domain of HP receptor family-defining regions is designated as a rectangular box crossed by a heavy bar representing the WSXWS motif. Sequences represented are rat NCAM (reference 79 as drawn in 56), IL-6 receptor (57), and IL-3 receptor (53).

these receptors, presumably contains the ligand binding site of the G-CSF receptor. The Ig and extra FN3-like domains of the G-CSF receptor may confer additional activities to this receptor, similar to the complex functions recognized for these domains in other cell surface molecules. These generally appear to involve participation in some form of cell recognition or adhesion. Both types of domains are capable of homotypic and heterotypic interactions (48, 69) that might lead to receptor self-association or binding to other proteins. Specific functions have been attributed to some type III repeats of fibronectin, including the binding of cells and heparin (70, 71) and heparin binding activity is also a property of at least one neural cell adhesion molecule (72). The potential for simultaneous recognition of heparin and G-CSF by the G-CSF receptor would have interesting functional implications, especially in light of the affinity of the heparin component of extracellular matrix for CSFs (73, 74). The particular response of a cell to G-CSF could thus depend on its adherence or that of G-CSF to the extracellular matrix. Alternatively it could allow G-CSF to mediate or specify interactions between cells and matrix, thus, directing margination or chemotaxis. The cloning of the human G-CSF receptor will provide reagents useful in the further elucidation of the biological roles of G-CSF and may allow development of new diagnostic or therapeutic agents.

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Address correspondence to Dr. Alf Larsen, Immunex Corp., Molecular Biology, 51 University St., Seattle, WA 98101. B.M. Curtis' present address is Oncogen, 3005 1st Ave., Seattle, WA 98101.

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Functional domains of the granulocyte colony-stimulating factor receptor

Rikiro Fukunaga, Etsuko Ishizaka-Ikeda,
Cai-Xi Pan, Yoshiyuki Seto and
Shigekazu Nagata

Osaka Bioscience Institute, 6-2-4 Furuedai, Suita-shi, Osaka 565,
Japan

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The granulocyte colony-stimulating factor (G-CSF) receptor has a composite structure consisting of an immunoglobulin(Ig)-like domain, a cytokine receptor-homologous (CRH) domain and three fibronectin type III (FNIII) domains in the extracellular region. Introduction of G-CSF receptor cDNA into IL-3-dependent murine myeloid cell line FDC-P1 and pro-B cell line BAF-B03, which normally do not respond to G-CSF, enabled them to proliferate in response to G-CSF. On the other hand, expression of the G-CSF receptor cDNA in the IL-2-dependent T cell line CTLL-2 did not enable it to grow in response to G-CSF, although G-CSF could transiently stimulate DNA synthesis. Mutational analyses of the G-CSF receptor in FDC-P1 cells indicated that the N-terminal half of the CRH domain was essential for the recognition of G-CSF, but the Ig-like, FNIII and cytoplasmic domains were not. The CRH domain and a portion of the cytoplasmic domain of about 100 amino acids in length were indispensable for transduction of the G-CSF-triggered growth signal. **Key words:** cytokine receptor family/granulocyte colony-stimulating factor/hemopoietic cells/signal transduction

Introduction

The proliferation and differentiation of hemopoietic cells such as granulocytes, macrophages, T cells and B cells are regulated by a family of cytokines including colony-stimulating factors (CSFs) and interleukins (ILs) (reviewed in Metcalf, 1989). Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein of 174 (human G-CSF) or 178 (murine G-CSF) amino acids, and specifically stimulates colony formation of neutrophilic granulocytes from bone marrow cells (Nagata *et al.*, 1986a,b; Tsuchiya *et al.*, 1986). G-CSF also stimulates proliferation of murine and human myeloid leukemia cells such as NFS-60 (Weinstein *et al.*, 1986), AML-193 (Santoli *et al.*, 1987) and OCI/AML 1 (Nara *et al.*, 1990) cells, while murine WEHI-3B D⁺ and 32DC13 cells can be induced by G-CSF to differentiate into granulocytes or monocytes (reviewed in Nicola, 1989; Nagata, 1990). G-CSF produced by stroma cells in bone marrow seems to play an essential role in maintaining the number of neutrophilic granulocytes in peripheral blood. G-CSF is produced also by macrophages stimulated with endotoxins (Metcalf and Nicola, 1985; Nishizawa and Nagata, 1990), and by fibroblasts or endothelial cells treated with TNF- α or IL-1 (Seelentag *et al.*, 1987; Koeffler *et al.*,

1988). The G-CSF thus accumulated in blood appears to be responsible for granulocytosis during the inflammatory process. Since administration of G-CSF to animals caused a marked granulopoiesis (Tsuchiya *et al.*, 1987), the substance is currently under clinical trials for patients suffering from granulopenia (Morstyn *et al.*, 1989).

Despite the biological importance of G-CSF, its mechanism of signal transduction has not been elucidated. A single class of high affinity receptor for G-CSF [dissociation constant (K_d) = 100–500 pM] is present on the precursor and mature cells of neutrophilic granulocytes as well as myeloid leukemia cells (Nicola and Peterson, 1986; Park *et al.*, 1989; Fukunaga *et al.*, 1990b). Purification of the G-CSF receptor from mouse myeloid leukemia NFS-60 cells has indicated that the receptor has an M_r of 100 000–130 000, and the monomeric form of this protein binds G-CSF with a low affinity (K_d = 2.6–4.2 nM), while its oligomeric forms show high affinity binding to G-CSF (K_d = 120–360 pM) (Fukunaga *et al.*, 1990b). Recently, we have isolated the cDNAs for murine (Fukunaga *et al.*, 1990a) and human G-CSF receptors (Fukunaga *et al.*, 1990c); expression of these receptor cDNAs in monkey COS cells gave rise to proteins which specifically bound G-CSF with a high affinity, suggesting that the single polypeptide encoded by the cDNA is sufficient to constitute the high affinity binding site for G-CSF.

Murine and human G-CSF receptors consist of 812 and 813 amino acids, respectively, and contain a single transmembrane domain (Fukunaga *et al.*, 1990a,c). In agreement with the fact that G-CSF has no species specificity between human and mouse, the amino acid sequences of human and murine G-CSF receptors have a considerable similarity (62.5% identity) (Fukunaga *et al.*, 1990c). In the extracellular domain (~600 amino acids) of the G-CSF receptor, there is a region of ~200 amino acids which shows significant homology to the receptors for IL-3–7, erythropoietin and GM-CSF, and the β -chain for the IL-2 receptor (Bazan, 1990a). In the case of the G-CSF receptor, this region (referred to as the cytokine receptor-homologous domain or CRH domain) is followed by a domain (~300 amino acids) consisting of three fibronectin type III (FNIII) modules, which is homologous to chicken contactin (Fukunaga *et al.*, 1990a). The cytoplasmic region of the G-CSF receptor has a limited similarity to that of the IL-4 receptor (Mosley *et al.*, 1989), and like other members of the cytokine receptor family, the region does not appear to contain the domain with kinase or other enzymatic activity. Very recently, the human cDNA for the IL-6 signal transducer, gp130, was isolated (Hibi *et al.*, 1990). The overall structure of gp130 is remarkably similar to that of the G-CSF receptor, and the similarity of human G-CSF receptor and gp130 is 46.3% on the amino acid sequence level, when conservative substitutions are included.

In order to investigate the role of the G-CSF receptor in the G-CSF-dependent signal transduction, we have expressed

the G-CSF receptor cDNA in various hematopoietic cell lines using a promoter of human elongation factor 1 α gene. The G-CSF receptor could function as a transducer for the G-CSF-triggered growth signal in IL-3-dependent FDC-P1 and BAF-B03 cells, but not in IL-2-dependent CTLL-2 cells. Utilizing this expression system, we have identified the functional domains of the G-CSF receptor.

Results

Establishment of cell lines expressing the G-CSF receptor

Mouse myeloid precursor cell line FDC-P1 and pro-B cell line BAF-B03 require IL-3 for their growth, whereas the

growth of murine cytotoxic T cell line CTLL-2 is strictly dependent on IL-2. In order to examine whether the cloned G-CSF receptor can transduce the G-CSF-triggered signal in these cells, we have established transformed cell lines expressing the G-CSF receptor cDNA. For this purpose, we took advantage of a recently constructed mammalian expression vector, pEF-BOS, which utilizes a constitutive promoter of the human polypeptide chain elongation factor 1 α (EF-1 α) gene (Uetsuki *et al.*, 1989), and which works very efficiently in various cell lines (Mizushima and Nagata, 1990). The pEF-BOS plasmid harboring a murine G-CSF receptor cDNA (p162) (Fukunaga *et al.*, 1990a) or human G-CSF receptor cDNA (pHQ3) (Fukunaga *et al.*, 1990c) was transfected into FDC-P1, BAF-B03 and CTLL-2 cells,

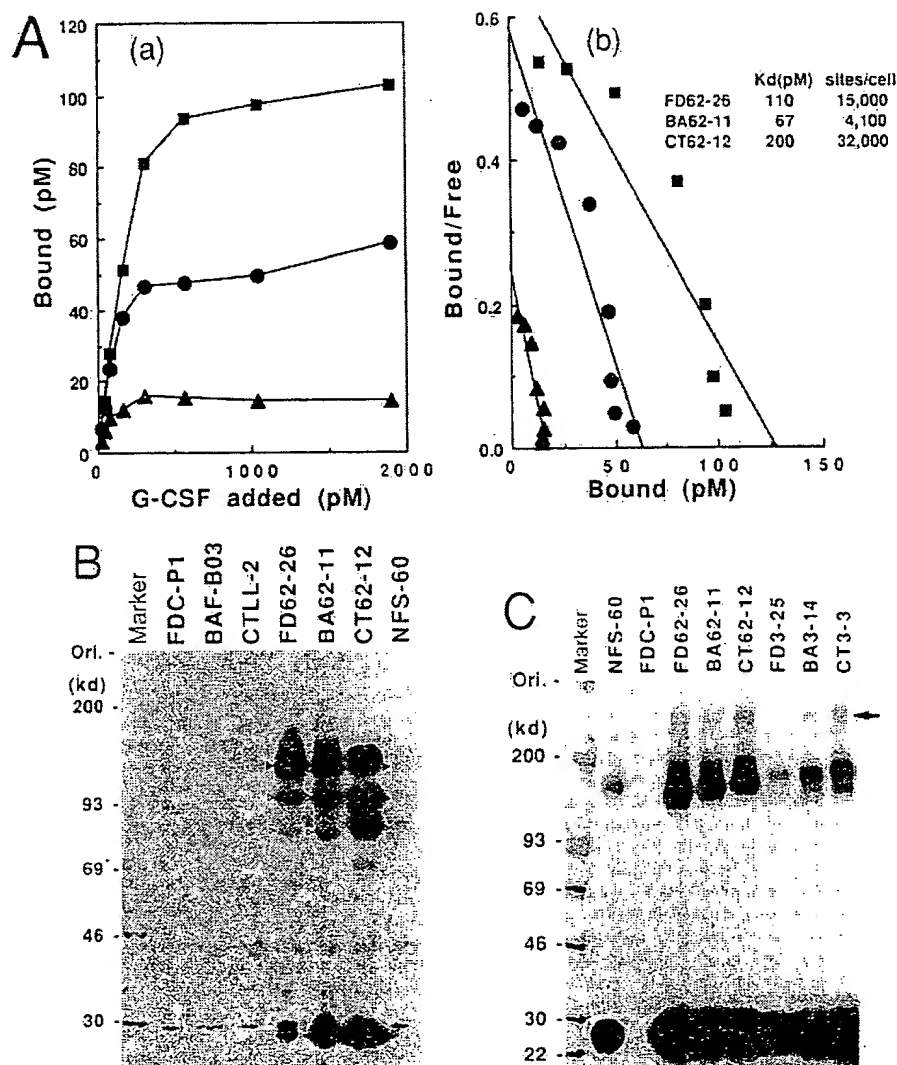


Fig. 1. Expression of mouse and human G-CSF receptor in hemopoietic cell lines. (A) G-CSF binding characteristics in transformant clones. (a) Saturation binding of $[^{125}\text{I}]\text{G-CSF}$ to transformant clones expressing mouse G-CSF receptor cDNA. The transformants derived from FDC-P1, BAF-B03 and CTLL-2 were designated FD62, BA62 and CT62, respectively. 2.5×10^6 cells/ml were incubated for 3 h at 4°C with various amounts of $[^{125}\text{I}]\text{G-CSF}$. The specific binding of $[^{125}\text{I}]\text{G-CSF}$ to FD62-26 (\bullet), BA62-11 (\blacktriangle) or CT62-12 (\blacksquare) was determined as the difference between total binding and non-specific binding which was measured in the presence of 500 nM unlabeled G-CSF. (b) Scatchard plot of G-CSF binding data shown in (a). (B) Immunoblotting of mouse G-CSF receptor with anti-MR9 serum. Cell lysates prepared from parental cell lines, their transformant clones and NFS-60 were analyzed by immunoblotting with an anti-mouse G-CSF receptor serum (anti-MR9) as described in Materials and methods. As size markers, ^{14}C -labeled molecular weight standards (rainbow marker, Amersham) were electrophoresed in parallel ('Marker' lane). The positions of the mature and less glycosylated G-CSF receptors are indicated by solid and open arrowheads, respectively. (C) Chemical cross-linking of the mouse and human G-CSF receptors with radioiodinated G-CSF. NFS-60, FDC-P1 and transformed cell clones (2×10^7 cells/ml) expressing mouse (FD62-26, BA62-11 and CT62-12) or human (FD3-25, BA3-14 and CT3-3) G-CSF receptor cDNA were incubated for 3 h at 4°C with 500 pM $[^{125}\text{I}]\text{G-CSF}$ and chemically cross-linked with disuccinimidyl suberate and disuccinimidyl tartrate as previously described (Fukunaga *et al.*, 1990b). The cell lysate (50 μg protein) was analyzed by SDS-PAGE, and the gel was dried and exposed to X-ray film.

together with a plasmid carrying the neomycin resistance gene. G418-resistant transformants were tested for the ability to bind [125 I]G-CSF, and several independent clones were isolated after limiting dilution from the binding-positive transformants.

The properties of the G-CSF binding to these stable transformants were first examined. The parental FDC-P1, BAF-B03 and CTLL-2 cells did not bind G-CSF (data not shown). On the other hand, the cell clones of FD62, BA62 and CT62, transformed with the mouse G-CSF receptor cDNA, bound G-CSF with high affinities ($K_d = 110$, 70 and 200 pM, respectively) (Figure 1A). The numbers of the G-CSF receptor expressed in clones of FD62 and BA62 were constantly 4000–15 000 sites per cell, while clones of CT62 expressed the mouse G-CSF receptor in the range 20 000–30 000 sites per cell. The transformants with the human G-CSF receptor cDNA (FD3, BA3 and CT3 cells) also expressed only a high affinity G-CSF receptor ($K_d = 170$ –300 pM) at 2400–20 000 sites per cell (data not shown). These K_d values are consistent with our previous results obtained with COS cells (Fukunaga *et al.*, 1990a,c) and confirm that the single polypeptide coded by the cloned G-CSF receptor cDNA is sufficient to constitute a high affinity binding site for G-CSF.

For the immunological detection of the G-CSF receptor, two proteins containing a portion of the extracellular domain (MR1, amino acids 36–326) or cytoplasmic domain (MR9, amino acids 631–812) of the mouse G-CSF receptor were

produced in *Escherichia coli*, and polyclonal antibodies against these proteins were prepared in rabbits. An immunoblot using anti-MR9 antiserum identified three molecular species varying in apparent molecular size (125–135 kDa, 105–110 kDa and 85–90 kDa) in the transformant clones as well as in mouse NFS-60 cells which express the endogenous G-CSF receptor (Figure 1B). The bands detected at ~30 kDa in the transformant clones are probably degraded forms of the receptor. The 125–135 kDa species seems to be the mature, cell surface receptor because the cross-linking of the cell surface receptor with radioactive G-CSF gave only a ligand–receptor complex with an apparent M_r of 150–160 kDa (Figure 1C). The other two species are likely to be less glycosylated or non-glycosylated intermediates. The small difference in molecular sizes of the receptor (Figure 1B and C) among the cell types could be due to difference in their glycosylation. Cross-linking experiments also confirmed that the transformant clones FD3-25, BA3-14 and CT3-3 expressed human G-CSF receptor (Figure 1C). We have often observed an additional cross-linked complex with higher molecular size in cells expressing a large number of G-CSF receptors (Figure 1C, indicated by an arrow). Since this complex has an M_r of ~300 kDa, it may represent the cross-linked dimer of the G-CSF receptor with which [125 I]G-CSF was cross-linked. The immunoblot analysis sometimes showed a faint band at 125–135 kDa in the parental FDC-P1, BAF-B03 and CTLL-2 cells (Figure 1B). This band seems to be a non-

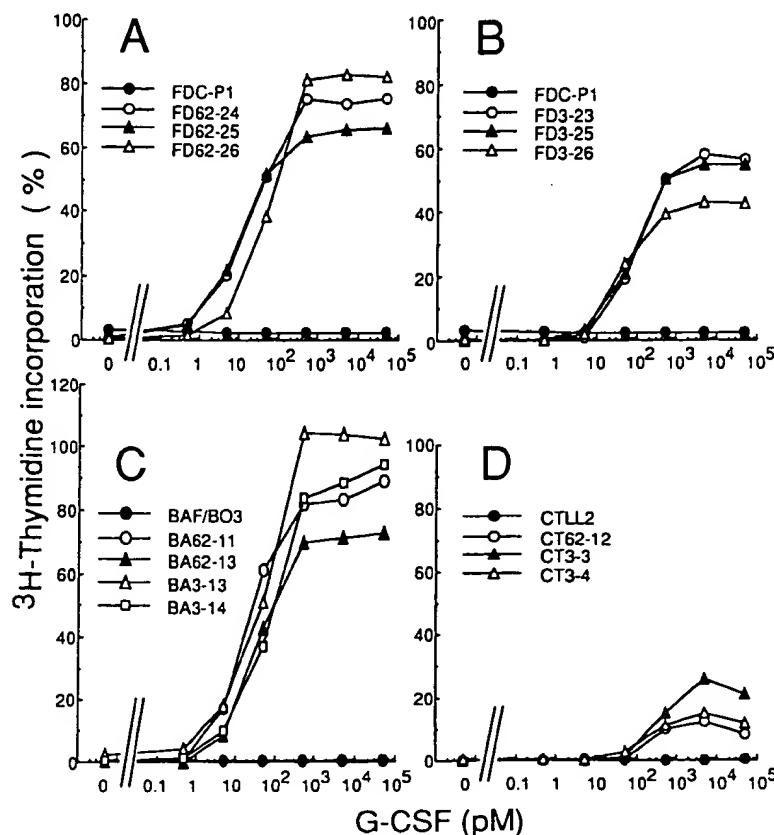


Fig. 2. G-CSF-dependent DNA synthesis of transformants expressing mouse or human G-CSF receptor cDNA. Parental cells and their transformed cell clones expressing mouse or human G-CSF receptor were cultured with 0–50 nM mouse G-CSF, and incorporation of [3 H]thymidine into cells was measured. The results are presented as the percentage of the maximum [3 H]thymidine incorporation observed with IL-3 (A, B and C) or IL-2 (D) in the respective cells. (A and B) FDC-P1 and its transformant clones expressing the mouse (A) or human (B) G-CSF receptor cDNA. (C) BAF-B03 and its transformant clones expressing mouse or human G-CSF receptor. (D) CTLL-2 and its transformant clones expressing mouse and human G-CSF receptor.

specific one because these cells neither bound G-CSF nor gave the cross-linked complex with [125 I]G-CSF (Figure 1C, data not shown).

G-CSF receptor can transduce the growth signal in FDC-P1 and BAF-B03 cells but not in CTLL-2 cells

The effect of G-CSF on the growth properties of the transformants expressing the G-CSF receptor was then examined by [3 H]thymidine uptake assay. As shown in Figure 2, none of the parental cell lines responded to G-CSF, whereas transformants derived from FDC-P1 and BAF-B03

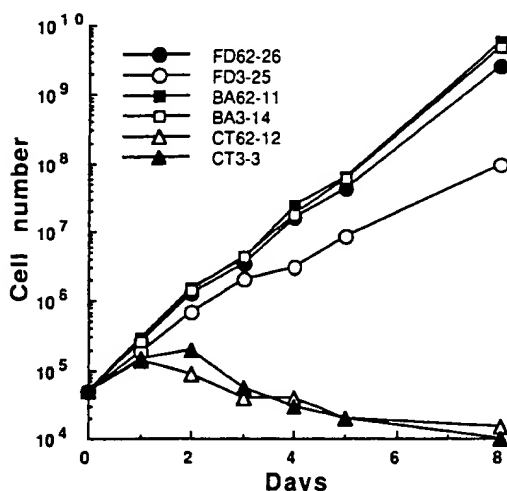


Fig. 3. G-CSF-dependent long-term growth of transformants expressing G-CSF receptor cDNA. Transformants growing in IL-3 or IL-2 were washed thoroughly and cultured at 5×10^4 cells/ml in RPMI1640 containing 10% FCS and 500 pM mouse G-CSF. Cell numbers were counted daily and cell concentrations were kept less than 10^6 cells/ml by appropriate dilution with the same medium.

responded to G-CSF in a concentration-dependent manner. In the FDC-P1 transformants, clones expressing murine G-CSF receptor (clones of FD62) responded slightly more efficiently than the clones expressing human G-CSF receptor (clones of FD3). The maximum [3 H]thymidine uptakes of FD62 and FD3 obtained at > 500 pM G-CSF were 70–80% and 50–60%, respectively, of that observed with an excess of murine IL-3 (Figure 2A and B). In BAF-B03 transformants, human and murine G-CSF receptors were equally effective, and the maximum response obtained at 500 pM of G-CSF was almost comparable to that observed with mouse recombinant IL-3 (Figure 2C). In accordance with the high responsiveness of these transformants to G-CSF, 500 pM G-CSF could support the long-term growth of clones of FD62, FD3, BA62 and BA3 in the absence of IL-3 (Figure 3). On the other hand, G-CSF stimulated very weakly the DNA synthesis of the CTLL-2 transformants (clones of CT62 and CT3) (Figure 2D). The response was $< 30\%$ of that observed with an excess of IL-2, and this weak responsiveness was insufficient to support the long-term proliferation or survival of these cells in the absence of IL-2 (Figure 3).

Construction and expression of cDNAs carrying various mutations in the subdomains of the G-CSF receptor

As shown in Figure 4, the G-CSF receptor contains a single transmembrane region which divides the molecule into two regions, the extracellular and the cytoplasmic. The extracellular region can be further divided into five subdomains, an immunoglobulin(Ig)-like domain, a CRH domain and three FNIII domains, based on homology with other proteins (Fukunaga et al., 1990a; Bazan 1990a) and the exon-intron organization of the G-CSF receptor gene (Y.Seto, R.Fukunaga and S.Nagata, unpublished results).

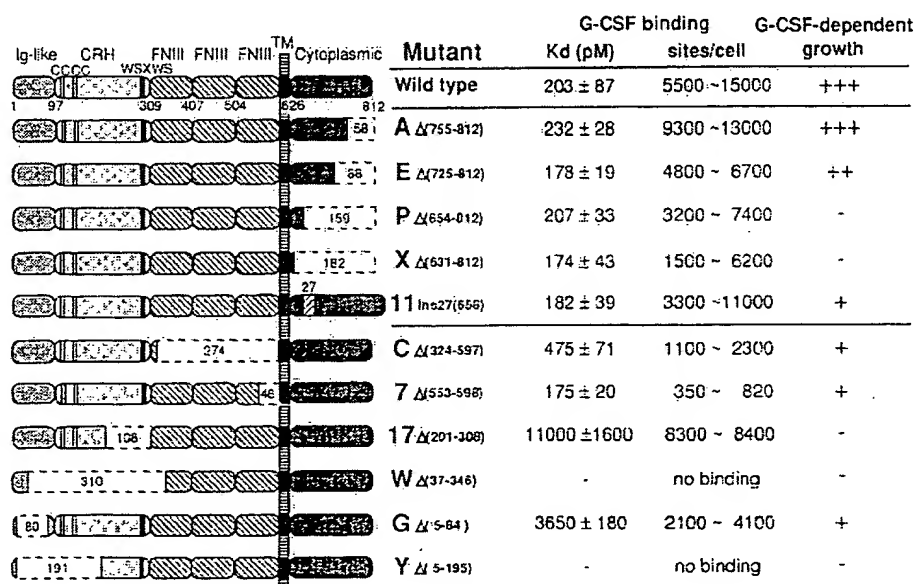


Fig. 4. Summary of mutational analysis of the G-CSF receptor. The extracellular domain of the G-CSF receptor is putatively divided into five subdomains, an Ig-like domain, a cytokine receptor-homologous (CRH) domain and three fibronectin type III (FNIII) domains (top). Four cysteine residues and a WSXWS motif conserved in the CRH domain are also indicated. The deletion mutants of mouse G-CSF receptor (mutants A, E, P, X, C, W, G and Y) and three variants (variants 11, 7 and 17), are shown schematically below the wild-type receptor. The portions deleted in the mutants are represented by the dotted lines and the number of amino acids deleted in each mutant is indicated. In all wild-type and mutant cDNAs, the coding region for the mature protein is preceded by an N-terminal signal sequence, which is not shown in the figure. The table at the right shows the G-CSF binding characteristics of the mutated receptor and their abilities to transduce the G-CSF-triggered growth signal.

In order to assign the functional domains of the G-CSF receptor, we have constructed a series of murine G-CSF receptor cDNAs which have various deletions in these subdomains. In the first set of deletion mutants (mutants A, E, P and X), the cytoplasmic region of the receptor was progressively deleted from the C-terminal end up to amino acid positions 754, 724, 653 and 630, respectively (Figure 4). For the extracellular region, four deletion mutants (C, W, G and Y) were constructed. In addition to these constructions, we have used as mutants variant receptors whose cDNAs were isolated from human and mouse cDNA libraries. The human G-CSF receptor variant (mutant 11) contains a 27 amino acid insertion between amino acid positions 657 and 658 in the cytoplasmic domain (Fukunaga *et al.*, 1990c), while murine variants carry the deletion of the C-terminal half of the third FNIII domain (mutant 7) or the C-terminal half of the CRH domain (mutant 17) (Figure 4). These G-CSF receptor variants seem to be generated by alternative splicing of the precursor RNA (Y.Itoh, E.Ishizaka-Ikeda, Y.Seto, R.Fukunaga and S.Nagata, unpublished results).

FDC-P1 cells were transformed with expression plasmids carrying the mutated cDNA as described above. Stable transformants expressing each mutant were isolated and designated FD-A, FD-E, FD-P and so forth. Expression of the mutated receptor in these transformants was first analyzed by immunoblot analysis using anti-MR9 antiserum. As shown in Figure 5A, the mature and less glycosylated receptor with molecular weights expected from the size of the deletion was observed in all transformant clones except

for clones of FD-P and FD-X, which were transformed with mutants containing little or no part of the MR9 polypeptide. A relatively weak intensity observed in mutants A and E was also due to deletion of parts of the cytoplasmic domain. An immunoblot with anti-MR1 antiserum recognizing a part of the extracellular domain of the mouse G-CSF receptor showed bands in transformant clones for all mutants except for the mutant W which had a deletion of the region recognized by anti-MR1 serum (Figure 5B). Furthermore, as shown in Figure 5C, cross-linking with [125 I]G-CSF gave a complex of the expected size in all transformant clones which bind G-CSF with a high affinity (Figure 4). In contrast, no cross-linked complex was observed in clones FD-17 (Figure 5C, lane 6), FD-G, FD-W and FD-Y (data not shown) which have a low or undetectable affinity to G-CSF (Figure 4). Expression of a variant of the human G-CSF receptor in FD11-10 cells was confirmed by the cross-linking experiment (Figure 5C). These results indicate that all mutants of the G-CSF receptor were expressed in the transformants, although the expression levels of mutants C, 7 and G, which have deletions in the FNIII or Ig-like domain, were relatively low in all clones so far tested (Figures 4 and 5).

The CRH domain is responsible for binding of G-CSF

Using the FDC-P1 transformants expressing the mutated G-CSF receptor, we have located the region of the receptor essential for binding of the ligand. Typical Scatchard plots of the G-CSF binding data with these transformants are shown in Figure 6, and summarized in Figure 4. The mutant

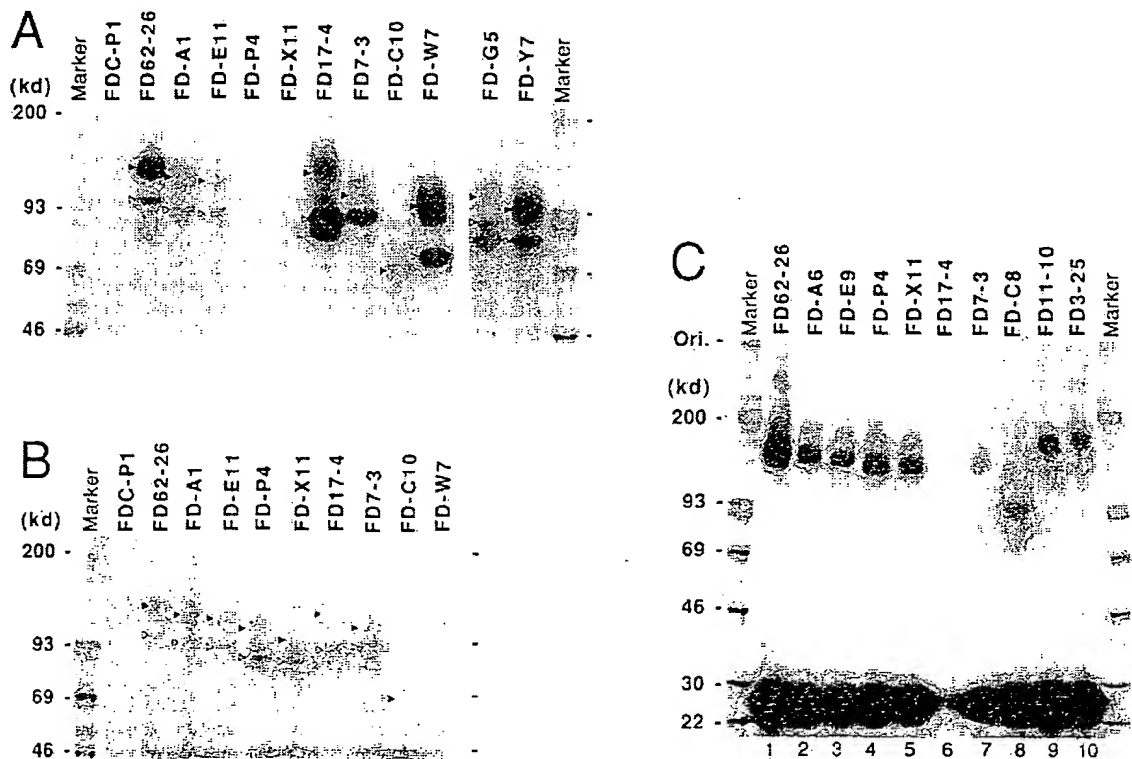


Fig. 5. Expression of G-CSF receptor mutants in FDC-P1 transformants. (A and B) Cell lysates of FDC-P1-derived transformants expressing wild-type or mutant cDNA of mouse G-CSF receptor were analyzed by immunoblotting with the anti-MR9 antiserum (A), or anti-MR1 antiserum (B) as described in Figure 1B. (C) The wild-type and mutant G-CSF receptors expressed in FDC-P1 transformants were cross-linked with [125 I]G-CSF and analyzed as described in Figure 1C. The cell lysate of 50 µg (lanes 1–5), 200 µg (lanes 6–8) or 100 µg (lanes 9 and 10) protein was electrophoresed.

X, containing only five amino acids in the cytoplasmic region, as well as mutants A, E, P and 11, could bind G-CSF with the same affinity as the wild-type receptor ($K_d \sim 200$ pM). These results indicate that the truncations or mutations in the cytoplasmic region do not affect the extracellular binding of G-CSF to the receptor.

Two mutants containing deletions in the FNIII domains (mutants 7 and C) could bind G-CSF with high affinities, although mutant C showed slightly lower affinity ($K_d = 480$ pM) than the wild-type receptor. On the other hand, mutant 17, which lacks the C-terminal 108 amino acids of the CRH domain, and mutant G, which lacks the Ig-like domain, bound G-CSF with ~ 50 - and 20-fold higher K_d values ($K_d = 11.0$ and 3.7 nM), respectively, than the wild-type receptor (Figures 4 and 6). The deletion either in the N-terminal half or in the entire region of the CRH domain (mutants Y and W) resulted in total inability to bind G-CSF. These results indicate that the N-terminal region of the CRH domain plays an essential role in the binding of G-CSF.

The CRH domain and part of the cytoplasmic region are necessary for signal transduction

We have then examined whether mutated G-CSF receptors are able to transduce the G-CSF-triggered growth signal into cells. Figure 7A shows the G-CSF dependent [3 H]thymidine uptake of the transformants expressing the mutated G-CSF receptor. Clones of FD-A and FD-E responded to G-CSF in a concentration-dependent manner, although the maximum response of FD-E clones was lower than that obtained with the wild-type G-CSF receptor. Clones of FD-A and FD-E could be maintained in the medium containing G-CSF instead of IL-3 (Figure 7B). On the other hand, G-CSF neither stimulated the DNA synthesis of FD-P and FD-X clones nor supported their long-term growth (Figure 7A and B). These results suggest that the 99 amino acid portion from position 626 to 724 in the cytoplasmic region is essential for transduction of the G-CSF-triggered growth signal into FDC-P1 cells. FD11 clones expressing the molecule which contains a 27 amino acid insertion responded weakly to G-CSF, and were able to grow slowly in the medium containing G-CSF.

G-CSF weakly stimulated [3 H]thymidine uptake of FD-7, FD-C and FD-G clones (Figure 7A) and these clones could be maintained in the medium containing G-CSF (Figure 7B), suggesting that neither Ig-like nor FNIII domains are

involved directly in signal transduction. As expected from the total inability of the mutants W and Y to bind G-CSF, these receptors could not respond to G-CSF at all. It may be noteworthy that a weak [3 H]thymidine uptake independent of G-CSF and IL-3 was observed in some clones of FD-C, FD-G and FD-Y cells (Figure 7A). Interestingly, mutant 17, which contained a deletion in the C-terminal half of the CRH domain, was completely inactive in signal transduction even in the presence of 50 nM G-CSF (Figure 7A), which should be sufficient for the binding of G-CSF to this mutant (Figures 4 and 6). These results suggest that the CRH domain of the receptor plays an essential role in signal transduction by G-CSF.

Discussion

G-CSF receptor is able to function as a growth signal transducer in IL-3-dependent cells but not in IL-2-dependent cells

To explore the signal transduction mechanism of the newly identified cytokine receptor family (Bazan, 1990b), it is essential to introduce the receptor cDNAs into various cells, especially hemopoietic cells. However, the expression of cDNA in hemopoietic cells is not an easy task. In this report, we have successfully used the promoter of human EF-1 α gene to express the G-CSF receptor cDNA in various hemopoietic cell lines (Figure 1). The expression level of the G-CSF receptor driven by the EF-1 α promoter in murine FDC-P1 cells was ~ 100 times greater than that driven by a CMV promoter (unpublished observation).

Expression of the G-CSF receptor in IL-3-dependent FDC-P1 or BAF-B03 cells enables these cells to grow in response to G-CSF (Figures 2 and 3). These results clearly indicate that the G-CSF receptor encoded by the cloned cDNA is sufficient to transduce the growth signal into cells, and suggest the presence of a common signal transducing pathway for the IL-3 and G-CSF systems. Introduction of protein kinases such as v-src, v-abl and v-fms into IL-3-dependent cells abrogated the dependence of cells on IL-3 (Cleveland *et al.*, 1989). Stimulation of cells with IL-3 induces the phosphorylation of tyrosine residues in a set of proteins, suggesting that some tyrosine kinase is involved in signal transduction by IL-3 (Koyasu *et al.*, 1987; Isfort *et al.*, 1988). Recently, Isfort and Ihle (1990) have shown that IL-3 and G-CSF stimulate tyrosine phosphorylation of the same protein (pp56) in NFS-60 cells. It is possible that the pp56 protein is one of the signal transducing molecules common in IL-3 and G-CSF systems.

Hatakeyama *et al.* (1989b) introduced the cDNA for the β -chain of IL-2 receptor into IL-3-dependent BAF-B03 cells. Since the BAF-B03 cells expressing IL-2 receptor could grow in the presence of IL-2, these authors postulated common signal transduction pathways in the IL-2 and IL-3 systems. However, G-CSF did not support the growth of the IL-2-dependent CTLL-2 cells transformed with the G-CSF receptor cDNA, though these cells expressed the high affinity G-CSF receptor in a large amount (Figure 1). These results may suggest that the signal transducing pathways downstream of the receptor are similar but different in the G-CSF and IL-2 systems, and that CTLL-2 cells are deficient in some components which are necessary for G-CSF-triggered signal transduction.

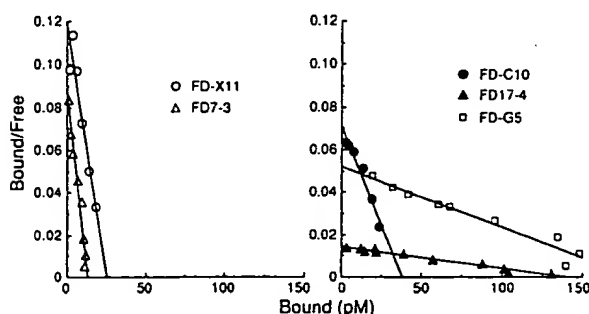


Fig. 6. Scatchard analysis of mutant G-CSF receptors expressed in FDC-P1 transformants. Cells of transformants FD-X11 (2.5×10^6 cells/ml), FD-C10, FD7-3, FD17-4 (10^7 cells/ml) and FD-G5 (2.7×10^7 cells/ml) were incubated with various concentrations of [125 I]G-CSF for 3 h at 4°C and specific binding was determined as described in Figure 1A.

Homodimer and heterodimer of the cytokine receptor

As shown in Figure 8, recent analyses on the structure of cytokine receptors and reconstitution of the receptors using their respective cDNAs have revealed that the receptors for IL-6, IL-2 and GM-CSF consist of two different subunits (Hatakeyama *et al.*, 1989a; Hibi *et al.*, 1990; Hayashida *et al.*, 1990). The α -chain of each receptor binds its ligand with low affinity, and the second chain (β -chain) is necessary for formation of the high affinity binding site and for

transduction of the signal. In contrast, the single polypeptide of the G-CSF receptor constituted a high affinity binding site for G-CSF, not only in hemopoietic cells (Figure 1A) but also in epithelial cells such as COS cells (Fukunaga *et al.*, 1990a,c) and C1271 cells (unpublished observation). Previously, we have shown that the monomer of the purified G-CSF receptor has a low affinity for G-CSF, whereas its dimer or oligomer constitutes a high affinity binding site for G-CSF (Fukunaga *et al.*, 1990b). Since the dissociation

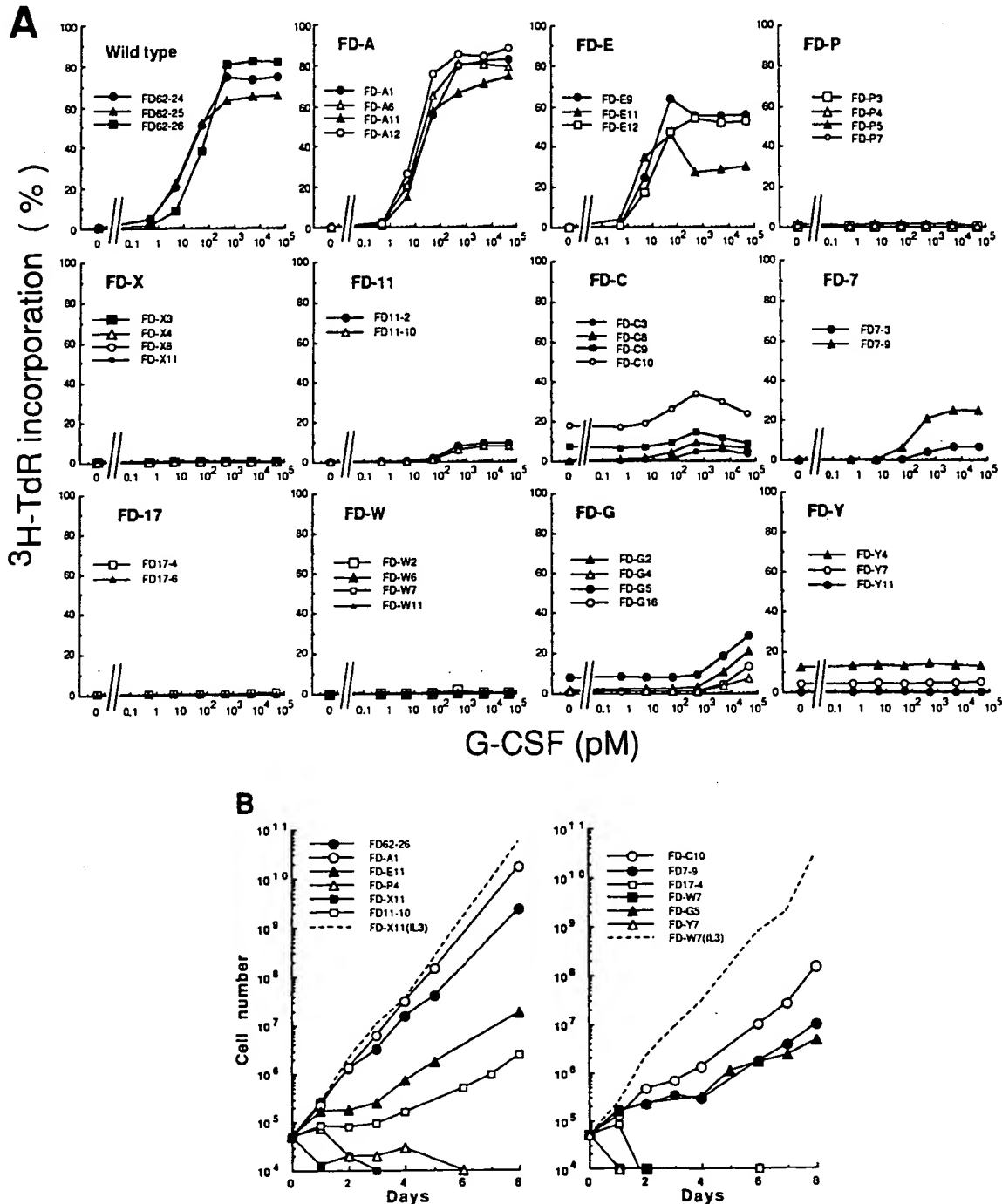


Fig. 7. G-CSF-dependent growth of transformants expressing mutant G-CSF receptor. (A) G-CSF-dependent [3 H]thymidine incorporation into cells. [3 H]thymidine incorporation was measured with several independent clones of individual FDC-P1 transformants expressing the mutant receptor as described in Figure 2. (B) G-CSF-dependent long-term proliferation of the transformants expressing mutant G-CSF receptor. Increase in cell number of FDC-P1 transformants expressing the mutant receptor in the presence of 20 nM (for FD-G transformant) or 500 pM (for other transformants) G-CSF was counted as described in Figure 3. Dotted lines indicate the growth curve of FD-X11 and FD-W7 cells in the presence of IL-3.

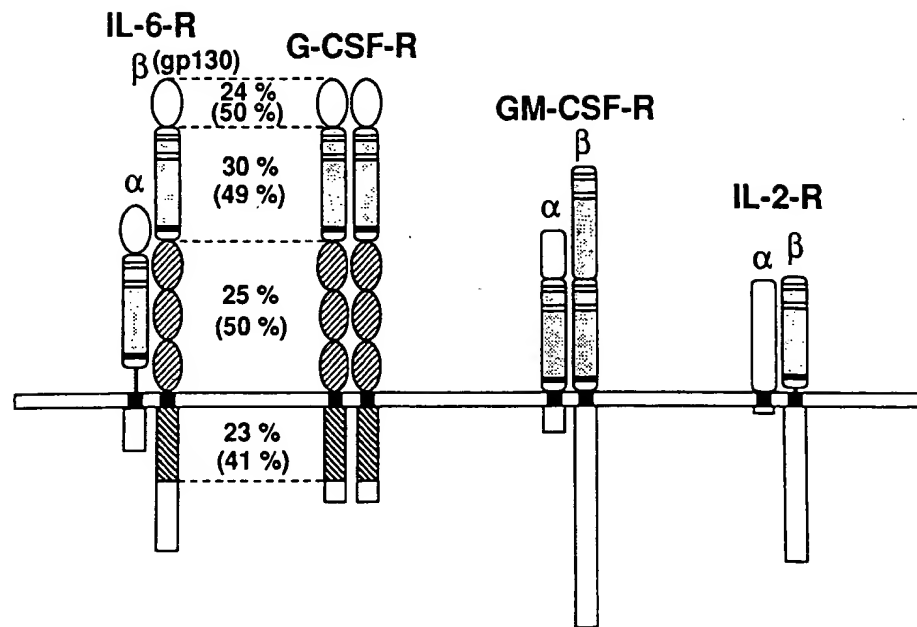


Fig. 8. Schematic representation of homo- and heterodimeric structures of functional, high affinity receptors for G-CSF, GM-CSF, IL-2 and IL-6. The CRH domains containing the conserved cysteine residues (thin bars) and the 'WSXWS' motif (thick bar) are indicated by shaded boxes. The gp130 protein (the β -chain of the IL-6 receptor) shows remarkable homology with the G-CSF receptor except for a C-terminal part of the cytoplasmic domain. The numbers indicate the percentage of identical amino acids in each subdomain. The numbers in parentheses show the percentage of homology including the conservative substitutions.

constant observed in intact cells is similar to that obtained with the dimer of the purified G-CSF receptor, it is likely that the G-CSF receptor exists as a dimer on the cell surface. Indeed, the cross-linking of the receptor with [125 I]G-CSF suggested the existence of a dimer of the receptor (Figure 1C). Availability of the antibody against the G-CSF receptor will make it possible to clarify the mechanism of the receptor dimerization in more detail.

Ligand binding domain of G-CSF receptor

Since the IL-4, IL-7 and erythropoietin receptors and the β -chain for the IL-2 receptor contain only the CRH domain in the extracellular region, it was postulated that this domain is involved in binding of the ligand (Bazan, 1990b). The present study using deletion mutants of the G-CSF receptor agrees with the above hypothesis. The receptor deleted in the C-terminal half of the CRH domain (mutant 17) could bind G-CSF with low affinity, while the mutants lacking the N-terminal region of the CRH domain (mutants W and Y) completely lacked the ability to bind G-CSF, suggesting that the N-terminal half containing the four conserved cysteine residues is indispensable for binding the ligand. Recently, Bass *et al.* (1991) have also shown that the hormone binding determinants of the growth hormone receptor is in the cysteine-rich region of the CRH domain.

The fact that mutants G and 17 bound G-CSF with low affinities suggests that the Ig-like domain and/or the C-terminal portion of the CRH domain may participate in the recognition of G-CSF, although it is possible that deletions in these domains simply caused some steric hindrance or conformational change in the ligand-binding site. An alternative possibility is that these domains are responsible for dimerization of the G-CSF receptor. The latter possibility may explain why these mutants showed low affinity binding to G-CSF with K_d values (3.7 and 11 nM) similar to those observed with the monomeric protein of the purified G-CSF

receptor (2.6–4.2 nM, Fukunaga *et al.*, 1990b). No cytokines showed a significant homology to G-CSF except for IL-6, which has a limited similarity to G-CSF (Nagata, 1990). Nevertheless, the CRH domain, which is the ligand-binding domain of the cytokine receptor molecule, showed a significant homology among these cytokines (Bazan, 1990b). Bazan (1990a) has recently proposed, based on the extrapolation from their primary structures, that these cytokines may have similar tertiary structure. Detailed mutational analysis of the ligand and the CRH domain, as carried out with growth hormone (Cunningham and Wells, 1989; Bass *et al.*, 1991), may reveal the mechanism through which different cytokines specifically recognize their own receptor.

Signal transducing domain of the G-CSF receptor

In the two mutants showing low affinity binding to G-CSF, the mutant G could respond to high concentrations of G-CSF, whereas the mutant 17 was totally inactive (Figures 4, 6 and 7). This result suggests that the C-terminal region of the CRH domain is indispensable for the signal transduction triggered by G-CSF. This C-terminal region, which contains the 'WSXWS' motif, may play a role in transducing some conformational change induced by the binding of G-CSF across the membrane to the cytoplasmic domain of the molecule. Alternatively, if the region deleted in the mutant 17 is involved in the formation of the dimeric receptor as discussed above, this result may imply that dimerization of the G-CSF receptor has a significance in signal transduction, as suggested in the cases of PDGF and EGF receptors (Heldin *et al.*, 1989; Bishayee *et al.*, 1989; Spaargaren *et al.*, 1991). In the [3 H]thymidine incorporation assay, the transformants expressing mutants G, C and 7 responded weakly to G-CSF (Figure 7A) but were able to grow slowly in the presence of G-CSF (Figure 7B). The Ig-like and FNIII domains, therefore, do not seem to play

Box1	
mGCSFR (630-643)	K T S F W S D V P D P A H S
hGCSFR (632-645)	K N P L W P S V P D P A H S
hIL6Rβ (648-461)	K K H I W P N V P D P S K S
mIL3R (473-486)	Y R K W K E K I P N P S K S
hGMCsFRβ (471-484)	R R K W E E K I P N P S K S
hIL2Rβ (249-262)	K K V L K C N T P D P S K F
hIL7R (249-262)	K P I V W P S I P D H K K T
v-mpl (177-190)	R H A L W P S I P D L H R V
mIL4R (235-248)	K K I W W D Q I P T P A R S
mEPOR (279-292)	Q Q K I W P G I P S P E S E
Box2	
mGCSFR(674-683)	I T E L E E D K K P
hGCSFR(673-683)	I T V L E E D E K K P
hIL6Rβ (693-703)	V V E I E A N D K K P
Box3	
mGCSFR(738-753)	P G V M Q V I R S D S T Q P L L
hGCSFR(739-754)	P G P G H Y L R C D S T Q P L L
hIL6Rβ(772-787)	P S V Q V F S R S E S T Q P L L

Fig. 9. The amino acid sequences conserved in the cytoplasmic domains of the G-CSF receptor and the β -chain (gp130) of the IL-6 receptor. The amino acid sequences of the corresponding regions of murine G-CSF receptor (mGCSFR, Fukunaga *et al.*, 1990a), human G-CSF receptor (hGCSFR, Fukunaga *et al.*, 1990c) and the β -chain of human IL-6 receptor (gp130) (hIL6R β , Hibi *et al.*, 1990) are aligned. The amino acid sequence of the Box 1 region of murine IL-3 receptor (Itoh *et al.*, 1990), the β -chain of human GM-CSFR (Hayashida *et al.*, 1990), the β -chain of human IL-2 receptor (Hatakeyama *et al.*, 1989a), human IL-7 receptor (Goodwin *et al.*, 1990), v-mpl (Souyri *et al.*, 1990), murine IL-4 receptor (Mosley *et al.*, 1989) and murine erythropoietin receptor (D'Andrea *et al.*, 1989) are also shown.

essential roles in signal transduction. Binding experiments using [125 I]G-CSF and Western blotting analysis have indicated that the numbers of the receptor expressed in these cells are lower than those in cells expressing the wild-type receptor (Figures 4 and 5). These results suggest that deletion of the FNIII domains may render the molecule unstable. The low response of these cells to G-CSF may be partly due to the poor expression of these mutant proteins.

The progressive deletion of the cytoplasmic region of the G-CSF receptor identified a region of 99 amino acids (positions 626-724) which seems to be essential for signal transduction by G-CSF in FDC-P1 cells. This region may constitute a domain of an unknown enzyme activity or associate with other molecules which transduce the signal. Previously, we have noticed that the cytoplasmic region of the G-CSF receptor has a similarity to that of the IL-4 receptor (Fukunaga *et al.*, 1990a). However, the homology between the cytoplasmic regions of the G-CSF receptor and the newly isolated gp130 (the β -chain) of the IL-6 receptor (Hibi *et al.*, 1990) is much more pronounced (Figure 8). As shown in Figure 9, three stretches of amino acid sequences (Boxes 1-3) are highly conserved between the G-CSF receptor and gp130, and two of the sequences (Boxes 1 and 2) are in the region which is critical for the signal transduction of the G-CSF receptor. IL-6 works on various cells including B cells, T cells and myeloid cells, and the gp130 seems to be responsible for the signal transduction triggered by IL-6 (Hibi *et al.*, 1990). The conserved region identified above may also be involved in signal transduction through the gp130 in the IL-6 system. In this regard, it may be noteworthy that the sequences similar to 'Box 1' can be found in the cytoplasmic region of receptors for IL-3, GM-CSF, IL-2, IL-7, IL-4 and erythropoietin, and in a recently identified oncogene v-mpl (Figure 9). In addition to the stimulation of cell proliferation, G-CSF and IL-6 have an

ability to induce cells to differentiate. Whether or not the region involved in transducing the differentiation signal is identical to the region for the growth signal remains to be studied.

Materials and methods

Cells and cell culture

Murine myeloid leukemia cell lines, NFS-60 cells (Weinstein *et al.*, 1986) and FDC-P1 cells (Dexter *et al.*, 1980) were kindly provided by Dr J.N. Ihle (St Jude Children's Research Hospital). Mouse pro-B cell line, BAF-B03 (Hatakeyama *et al.*, 1989b) and mouse cytotoxic T cell line, CTLL-2 (ATCC TIB 214) were provided by Drs M. Hatakeyama and T. Taniguchi (Institute for Molecular and Cellular Biology, Osaka University). NFS-60, FDC-P1 and BAF-B03 cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS, Hyclone) and 10-20 U/ml of recombinant mouse IL-3 (Fukunaga *et al.*, 1990b). CTLL-2 cells were grown in RPMI1640 medium containing 10% FCS and 10 ng/ml recombinant human IL-2 which was kindly provided by Dr H. Matsui (Ajinomoto Co., Tokyo).

Plasmid construction

Plasmid p162 carrying the full length cDNA for mouse G-CSF receptor in CDM8 vector has been described previously (Fukunaga *et al.*, 1990a). Two variant cDNAs, pG17 and pF1, were obtained from the NFS-60 cDNA library (Fukunaga *et al.*, 1990a) by colony hybridization with the cDNA of p162 as a probe, and will be described elsewhere in detail. The *Xba*I cDNA fragment was excised from p162, pG17 and pF1, and inserted into the *Xba*I site of a mammalian expression vector pEF-BOS (Mizushima and Nagata, 1990) to produce pBOS-I62 (wild-type receptor), pBOS-G17 (variant 17) and pBOS-JF7 (variant 7). The expression plasmid for the wild-type human G-CSF receptor (pHQ3) and its variant 11 (pQW11) has been described previously (Fukunaga *et al.*, 1990c).

For the construction of mutants containing the deletion in the cytoplasmic domain of mouse G-CSF receptor, p162 was digested with either *Xmn*I (at 2140), *Bsp*HI (at 2209), *Bst*EII (at 2421) or *Apa*I (at 2516), and if necessary, ends were blunted with the Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase. An *Xba*I linker (CTCTAGAG), which allows the addition of a leucine residue followed by an in-frame termination codon, was ligated to the blunt-ended DNA and digested with *Xba*I. The *Xba*I fragments containing the G-CSF receptor cDNA were then inserted into pEF-BOS to generate pBOSdXmn (mutant X), pBOSdBsp (mutant P), pBOSdBstE (mutant E), and pBOSdApa (mutant A).

To construct pBOSdWS (mutant W), pBOS-I62 was digested with *Kpn*I (at 1829) and *Bgl*II (at 358), and the *Bgl*II-*Kpn*I DNA fragment derived from the cDNA was digested with *Xho*II (at 1223 and 1288). The *Xho*II-*Kpn*I DNA fragment was separated using agarose gel electrophoresis and inserted into pBOS-I62, which was digested with *Kpn*I (at 1829) and partially digested with *Bgl*II (at 358). For the construction of pBOSdCON (mutant C), p162 was digested with *Eco*T22I (at 1106) and *Bsp*HI (at 2209), and the *Eco*T22I-*Bsp*HI cDNA fragment was digested with *Xho*II (at 1223, 1288 and 2045). The 117 bp *Eco*T22I-*Xho*II and the 164 bp *Xho*II-*Bsp*HI DNA fragments were then ligated with p162 digested with *Eco*T22I and *Bsp*HI. The *Xba*I DNA fragment of the resultant plasmid was inserted into pEF-BOS to produce pBOSdCON. To construct pBOSdIg (mutant G), the cDNA fragment of p162 was digested with *Taq*I (at 265, 838 and 2000), treated with Klenow fragment and digested with *Hind*III (at 162) to yield a 103 bp *Hind*III-*Taq*I (blunt) fragment. In addition to this fragment, the cDNA fragment of p162 was digested with *Tth*111I (at 297, 501 and 1938), blunt-ended and digested with *Kpn*I (at 1829). The 103 bp *Hind*III-*Taq*I (blunt) fragment and the 1.33 kb *Tth*111I (blunt)-*Kpn*I fragment were ligated together with a 6.3 kb *Hind*III-*Kpn*I fragment, which was prepared by digestion of pBOS-I62 with *Kpn*I (at 1829) and *Hind*III (partially at 162) to produce pBOSdIg. To construct pBOSdICy (mutant Y), the cDNA fragment of p162 was digested with *Taq*I (at 265, 838 and 2000), *Hind*III (at 162) and *Kpn*I (at 1829); the 103 bp *Hind*III-*Taq*I fragment and 991 bp *Taq*I-*Kpn*I fragment were then ligated with the 6.3 kb *Hind*III-*Kpn*I fragment described above. All constructions were confirmed by restriction enzyme mapping and DNA sequencing analysis.

Transfection of DNA

Cells were transfected with plasmid DNAs by electroporation (Potter *et al.*, 1984). The electroporation was carried out using Gene Pulser (BioRad) essentially according to the manufacturer's instructions. In brief, 8×10^6 cells were suspended in 0.8 ml of phosphate-buffered sucrose [7 mM sodium phosphate buffer (pH 7.4), 270 mM sucrose and 1 mM $MgCl_2$]. Eighty micrograms of the G-CSF receptor expression plasmid which had been

linearized by digestion with *Apa*LI, and 2 µg of *Xho*I-digested pSTneoB (Kato et al., 1987) were added to the cell suspension, which was incubated on ice for 10 min. Cells were exposed to a 350 V pulse with a capacitance of 25 µF, and returned to ice. After incubation on ice for 10 min, cells were diluted with 50 ml of RPMI 1640 medium/10% FCS containing IL-3 or IL-2, and cultivated in 24-well plates. Transfected cells were selected by culturing cells in medium containing G-418 at a final concentration of 0.5 mg/ml (for FDC-P1, and CTLL-2) or 2 mg/ml (for BAF-B03). Subcloning of the transfected cells was carried out by limiting dilution.

Cell proliferation assay, binding of G-CSF and chemical cross-linking

1.5×10^4 cells (100 µl) were mixed with various concentrations of G-CSF, IL-3 or IL-2 in 96-well microtiter plates. After incubation at 37°C for 22 h, 0.5 µCi of [³H]thymidine (specific activity, 74 GBq/mmol) was added per well and further incubated for 4 h at 37°C prior to harvest.

Radioiodination of murine recombinant G-CSF, binding of [¹²⁵I]G-CSF to cells, and chemical cross-linking were performed as described previously (Fukunaga et al., 1990a,b).

Preparation of antibodies against murine G-CSF receptor

Two different anti-mouse G-CSF receptor sera (anti-MR1 and anti-MR9) recognizing either a portion of the extracellular domain (amino acid positions 36–326) or the cytoplasmic domain (positions 631–812) were prepared as follows. The corresponding regions of murine G-CSF receptor were first produced in *E. coli* using the expression system developed by Studier et al. (1990). The expression vector pGEMEX-1 (Promega) was digested with *Nhe*I and *Bam*HI and the ends were filled in using the Klenow fragment. The DNA was religated to generate pEX, in which the *phi*10 gene was deleted and the *Bam*HI site was placed immediately downstream of an ATG initiation codon. To express the extracellular portions of murine G-CSF receptor, the 865 bp *Xho*II DNA fragment (nucleotide position 358–1223) of p162 was inserted into the *Bam*HI site of pEX to generate pEX-MR1. To express the cytoplasmic region of murine G-CSF receptor, pGEX-MR9 was constructed by ligating the 900 bp *Xmn*I–*Sph*I DNA fragment (nucleotides 2140–3126) of pJ17 (Fukunaga et al., 1990a) with the *Hinc*II and *Sph*I-digested pGEMEX-1 vector.

E. coli BL21(DE3)pLysS (Studier et al., 1990) was transformed with pEX-MR1 or pGEX-MR9, and the relevant products were purified essentially according to the method described by Sambrook et al. (1989). When the cell lysates were centrifuged, most of the recombinant proteins were found in inclusion bodies of the precipitate. After successive washing with H₂O and 0.1 M Tris–HCl (pH 8.5) containing 2 M urea, the precipitates were dissolved in a solubilizing buffer [50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl containing 8 M urea and 0.1 mM APMSE]. The G-CSF receptor polypeptides were purified by gel filtration on an Aca 54 column which was equilibrated with the solubilizing buffer, and dialyzed against phosphate-buffered saline (PBS). The purified proteins, which were >90% pure, were used to immunize rabbits to obtain the antisera.

Immunoblot analysis

About 2×10^7 cells collected by centrifugation were suspended in 100 µl of PBS containing a mixture of protease inhibitors (Fukunaga et al., 1990b), lysed by adding an equal vol of 2× sample buffer [0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 5% 2-mercaptoethanol] and sonicated to shear genomic DNA. After heating at 95°C for 5 min, the samples were electrophoresed on a 4–20% gradient polyacrylamide gel. Electrophoretic transfer of proteins to a GVHP membrane filter (Millipore) was performed as described previously (Fukunaga et al., 1990b). The blotted filter was rinsed with Block Ace (Dainippon Seiyaku, Co., Japan) for 60 min at 37°C, washed three times with PBS and once with TPBS (PBS containing 0.1% Tween 20), and incubated with 10 ml of TPBS containing 10% Block Ace and 1 µl of anti-MR1 serum or 0.1 µl of anti-MR9 serum for 60 min at room temperature. The filter was then washed three times with TPBS and incubated with 10 ml of TPBS containing 10% Block Ace and 18.5 kBq/ml [¹²⁵I]-labeled F(ab')₂ fragment of donkey anti-rabbit Ig antibody (Amersham). After incubation for 60 min at room temperature, the filter was washed six times for 10 min each with TPBS, dried and subjected to autoradiography.

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the injection of exogenous RvA obtained from electrically excitable tissues into *Xenopus* oocytes causes the appearance of voltage-operated Ca^{2+} channels in the oocyte membrane. These channels are distinct from the endogenous ones in respect to their time course and inactivation properties. Moreover, heart and brain RNA each encode at least two distinct types of Ca^{2+} channels. For the heart RNA, the slow current appears to show the appropriate sensitivity to and modulation by transmitters and intracellular messengers. Significantly, a rapidly inactivating, norepinephrine- and dihydropyridine-insensitive Ca^{2+} current has recently been reported in heart cells (10).

The injection of RNA from various tissues into *Xenopus* oocytes should aid in the characterization of different types of Ca^{2+} channels. Such data will complement experi-

mental neurons, cell lines, and reconstituted membranes. If specific RNA populations are used, it may also be possible to clarify the relations among components of Ca^{2+} channels and the interactions between channels and modulatory elements.

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Sequence and Expression of Human Estrogen Receptor Complementary DNA

GEOFFREY L. GREENE, PAUL GILNA, MICHAEL WATERFIELD, ANDREW BAKER, YVONNE HORT, JOHN SHINE

The mechanism by which the estrogen receptor and other steroid hormone receptors regulate gene expression in eukaryotic cells is not well understood. In this study, a complementary DNA clone containing the entire translated portion of the messenger RNA for the estrogen receptor from MCF-7 human breast cancer cells was sequenced and then expressed in Chinese hamster ovary (CHO-K1) cells to give a functional protein. An open reading frame of 1785 nucleotides in the complementary DNA corresponded to a polypeptide of 595 amino acids and a molecular weight of 66,200, which is in good agreement with published molecular weight values of 65,000 to 70,000 for the estrogen receptor. Homogenates of transformed Chinese hamster ovary cells contained a protein that bound [3H]estradiol and sedimented as a 4S complex in salt-containing sucrose gradients and as an 8 to 9S complex in the absence of salt. Interaction of this receptor- $[^3H]$ estradiol complex with a monoclonal antibody that is specific for primate ER confirms the identity of the expressed complementary DNA as human estrogen receptor. Amino acid sequence comparisons revealed significant regional homology among the human estrogen receptor, the human glucocorticoid receptor, and the putative *v-erbA* oncogene product. This suggests that steroid receptor genes and the avian erythroblastosis viral oncogene are derived from a common primordial gene. The homologous region, which is rich in cysteine, lysine, and arginine, may represent the DNA-binding domain of these proteins.

THE REGULATION OF GENE EXPRESSION in eukaryotic cells by estrogens and other steroid hormones involves the interaction of specific intracellular receptor proteins with the genome, resulting in the activation of selected sets of responsive genes (1). As a consequence, DNA synthesis in certain target cells is altered, and there are changes in the synthesis of specific RNA's and proteins involved in the regulation of cell proliferation, differentiation, and physiologic function in diverse tissues. In addition, steroid hormones and their receptors appear to be involved in the regulation of

abnormal growth in various tumors and tumor cell lines (2). Recent data from several laboratories (3) suggest that steroids may exert their effects by binding directly to an intranuclear receptor molecule that is weakly associated with nuclear components in the absence of ligand. Binding of hormone to its receptor results in conversion of the receptor-steroid complex to a form that associates with high affinity to one or more nuclear components. The molecular nature of this association and of the subsequent modulation of specific gene transcription is not known, although a number of nuclear accep-

tor sites have been proposed. These include specific DNA sequences (4), the nuclear matrix (5), and acidic nonhistone protein-DNA complexes (6). Although distinct steroid- and DNA-binding domains have been postulated to exist in all steroid receptors, few data are available on the detailed structure, composition, and chemical properties of the subunit that binds both steroid and DNA, and virtually nothing is known about the possible involvement of other components that do not bind steroid.

Determination of the primary structure of the estrogen receptor (ER) and expression of this molecule in homologous and heterologous systems can provide valuable information about structure-function relationships at a molecular level. Although ER is distributed in a tissue-specific manner, many of these cell types also express receptors for several other steroid hormones (7). Thus, it is likely that the specificity of control of responsive elements by steroids is determined, at least in part, by the primary structure of the receptor protein. Like other steroid receptors, hormone-occupied ER appears to recognize discrete DNA sequences that are generally upstream of transcriptional start sites in responsive genes. In the prolactin gene, footprinting analysis revealed a specific binding site for ER about 2 kb upstream of the start site (8); similar analyses of genes responsive to progestins and glucocorticoids revealed binding sites

G. L. Greene and P. Gilna, Ben May Laboratory for Cancer Research, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637.
M. Waterfield, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom.
A. Baker, Y. Hort, J. Shine, California Biotechnology Inc., Palo Alto 94304.

sequences were identified in randomly primed λ gt10 and λ gt11 MCF-7 cDNA libraries by screening either with monoclonal ER antibodies or with synthetic oligonucleotides corresponding to two peptide sequences obtained from purified MCF-7 human ER. Among the cDNA clones isolated

at nucleotides -54 to -52 suggests that it may contain non-ER fusion sequence resulting from a cloning artifact. The sequencing strategy was as follows: OR8 cDNA was subcloned into the Eco RI site of M13mp9. Clones containing both orientations of the cDNA were isolated and subjected to DNA sequence analysis by the method of Sanger *et al.* (13), with a series of specific oligonucleotide primers homologous to either the adjacent M13 sequence or to previously determined cDNA sequences.

by oligonucleotide hybridization was a 2.1-kb cDNA (OR8) that cross-hybridized with all other cDNA's and that contained the expected sequences for the two ER peptides. In addition, this cDNA hybridized selectively to a 6.2-kb polyadenylated RNA which, when translated *in vitro* in the presence of [35 S]methionine, coded for the synthesis of immunoreactive 65-kD ER, as well as for a smaller amount of an immunoreactive 46-kD protein (10). The molecular size of the major product is similar to published values of 65-67 kD for ER from several sources

(11). The identity of the smaller peptide is not known, but it may represent an *in vitro* degradation product of the 65-kD ER. The size of the mRNA for ER indicates that it is likely that a large portion is not translated. A 65-kD protein would require about 1.8 kb of coding sequence; this would leave more than 4 kb untranslated. This untranslated region is likely to be at the 3' end of the gene, as found in several other receptor mRNA's, including the human glucocorticoid receptor mRNA, which also contains a long 3'-untranslated region (12).

The 2.1-kb OR8 cDNA insert was the only cDNA long enough to contain the entire coding sequence for a 65-kD protein. Since this insert cross-hybridizes with all of the cloned cDNA's, regardless of selection method (10), OR8 should correspond to most or all of the MCF-7 human ER. To test this, the nucleotide sequence of this clone was determined by the method of Sanger *et al.* (13) according to the strategy described in Fig. 1. An open reading frame of 1785 nucleotides is the sequence encoding the human estrogen receptor, and corresponds to 595 amino acids and a calculated molecular weight of 66,200. The translation initiation site was assigned to the methionine codon at nucleotides 1 to 3 because this ATG triplet is the first to appear downstream from the in-frame terminator TGA at nucleotides -54 to -52. Although the actual initiation site has not been unequivocally established, amino acid sequence obtained from a cyanogen bromide fragment of MCF-7 ER corresponds to residues 12 to 26 (Fig. 1), which is very close to the proposed start site. The codon specifying the valine at position 595 is followed by a TGA translation termination codon. Peptide sequences from purified ER occur throughout the proposed open reading frame, including one that corresponds to residues 543 to 560 near the carboxyl terminus of the polypeptide (Fig. 1). Thus, all of the coding sequence for MCF-7 human ER is present in the OR8 cDNA insert.

To determine whether the OR8 cDNA would code for the synthesis of functional human ER in a heterologous cell system, OR8 was inserted into a pBR vector containing a metallothionein promoter and SV40 enhancer sequence (14), as described in Fig. 2. This OR8-containing plasmid was then used to transform Chinese hamster ovary cells (CHO-K1) (15), and cell homogenates were analyzed for the expression of human ER in a form capable of binding estradiol. Sedimentation analysis of a low-salt extract labeled with [3 H]estradiol revealed the presence of a receptor-estradiol complex which sedimented at 8-9S in 10 mM KCl (Fig. 2A) and at 4S in 0.4M KCl (Fig. 2B). This complex reacted with three different monoclonal ER antibodies to form 8S immune complexes (Fig. 2B). One of these antibodies is specific for primate ER (D75P3y) (16, 17). [3 H]Estradiol-binding in both the 4S and 8S complexes was abolished by the addition of a 200-fold molar excess of nonradioactive estradiol (Fig. 2B) or diethylstilbestrol. Extracts of untransformed CHO-K1 cells did not contain receptor-estradiol complexes (Fig. 2A). In addition, the concentration of the receptor-estradiol complex was more than

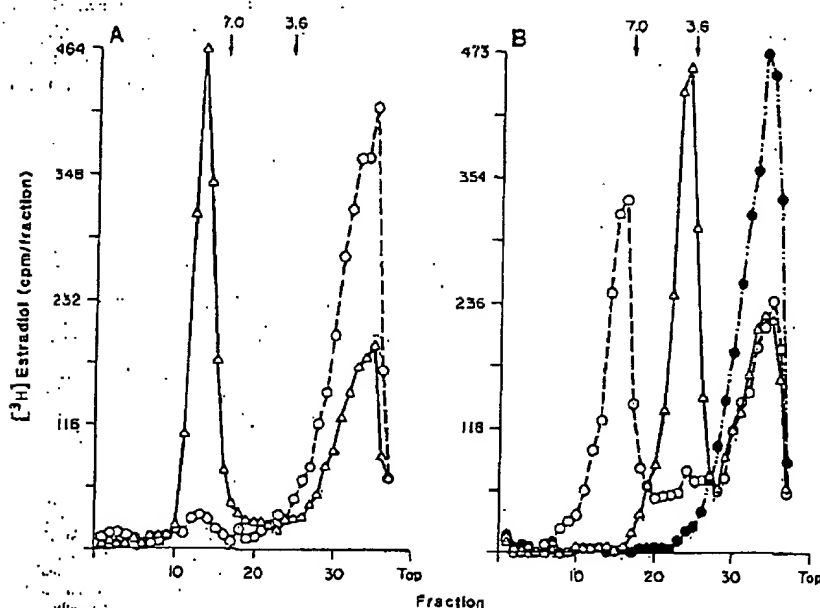


Fig. 2. Sedimentation analysis of human estrogen receptor expressed by OR8 cDNA in CHO-K1 cells and labeled with [3 H]estradiol. (A) Sedimentation profiles in low-salt gradients (10 mM KCl) of OR8-expressed human ER (Δ - Δ) or extract from untransformed CHO-K1 cells (\bullet - \bullet) labeled with [3 H]estradiol (0.5 nM). (B) Sedimentation profiles in high-salt gradients (400 mM KCl) of expressed ER labeled with [3 H]estradiol (0.5 nM) and incubated with (O-O) or without (Δ - Δ) D75P3y immunoglobulin G (IgG), and expressed ER labeled with [3 H]estradiol (0.5 nM) plus unlabeled estradiol (100 nM) (\bullet - \bullet). Untransformed CHO-K1 cells and cells transformed with the expression vector described below were grown in monolayer culture to confluency in a zinc-supplemented Dulbecco's minimal essential medium plus Ham's F-12 medium (22). After removal from the substrate with EDTA, the cells were homogenized by Polytron disruption in a buffer containing 10 mM Tris (pH 7.4) and 20 mM sodium molybdate (16). Homogenates were centrifuged at 253,000g for 30 minutes and the supernatant fractions were labeled with 0.5 nM [3 H]estradiol (57 Ci/mmol), with or without a 200-fold molar excess of nonradioactive estradiol, for 60 minutes at 4°C. Experiments with excess diethylstilbestrol in place of estradiol showed the same pattern of radioactivity on the gradient. In a separate experiment, aliquots (200 μ l) of labeled extract were incubated for 60 minutes at 4°C either in the presence or absence of rat monoclonal ER antibody (10 μ g of D75P3y, D547Sp, or H222Sp) in a final volume of 220 μ l. For sedimentation analyses, aliquots (200 μ l) of labeled extract or incubation mixture were layered onto linear 10 to 30 percent sucrose gradients (3.5 ml), prepared in 10 mM Tris, 10 mM sodium molybdate, 1.5 mM EDTA, pH 7.4, and either 10 mM KCl (low salt) or 400 mM KCl (high salt), and centrifuged at 0°C for 15 hours at 253,000g. Successive 100- μ l fractions were collected and radioactivity was measured in Triton X-100 toluene scintillation mixture at 35 percent counting efficiency. [14 C]Ovalbumin (3.6S) and [14 C]IgG (7.0S) were used as sedimentation markers in parallel gradients; their positions are designated by arrows. The OR8 expression vector was constructed by subcloning the OR8 cDNA into pMTpn (14). This vector consists of a 870-bp Hind III-Bam HI fragment from the human metallothionein II gene (14) inserted into the polylinker region of pUC9. This genomic fragment contains metal regulatory regions, glucocorticoid-receptor binding sites, and the promoter, transcription start, and 5'-untranslated region of the metallothionein II gene. To provide for transcription stop and polyadenyl addition signals, the 600-kb Sma I-Eco RI fragment from the 3' end of the human growth hormone gene was placed on the 3' side of the OR8 cDNA. The vector also contains the SV40 origin of replication and enhancer sequences immediately 5' to the metallothionein promoter.

h-GR	405	pro	pro	ser	ser	ser	thr	ala	thr	thr	gly	pro	pro	pro	lys	leu
h-ER	169	asn	asp	lys	gly	ser	met	ala	met	glu	ser	ala	lys	glu	thr	arg
v-erbA	21	ser	ser	met	gly	tyr	ile	pro	ser	cys	leu	asp	lys	asp	glu	gln
h-GR	421	cys	leu	val	cys	ser	asp	glu	ala	ser	gly	cys	his	tyr	gly	val
h-ER	185	cys	ala	val	cys	asn	asp	tyr	ala	ser	gly	tyr	his	tyr	gly	val
v-erbA	37	cys	val	val	cys	gly	asp	lys	ala	thr	gly	thr	his	tyr	arg	cys
h-GR	437	thr	cys	gly	ser	cys	lys	val	pho	phe	lys	arg	ala	val	glu	gly
h-ER	201	ser	cys	glu	gly	cys	lys	ala	phc	phc	lys	arg	ser	ile	glu	gly
v-erbA	53	thr	cys	glu	gly	cys	lys	ser	phc	phc	arg	arg	thr	ile	glu	lys
h-GR	452	gln	his	asn	---	tyr	leu	cys	ala	gly	arg	asp	asp	cys	ile	ile
h-ER	216	---	his	asn	asp	tyr	met	cys	pro	ala	thr	asn	gly	cys	thr	ile
v-erbA	69	leu	his	pro	thr	tyr	ser	cys	thr	tyr	asp	gly	cys	cys	val	ile
h-GR	467	lys	ile	arg	arg	lys	asn	cys	pro	ala	cys	arg	tyr	arg	lys	cys
h-ER	231	lys	asn	arg	arg	lys	ser	cys	glu	ala	cys	arg	leu	arg	lys	cys
v-erbA	85	lys	ile	thr	arg	asn	gln	cys	gln	leu	cys	arg	phe	lys	lys	cys
h-GR	483	gln	ala	gly	met	asn	leu	glu	ala	arg	lys	thr	lys	lys	lys	ile
h-ER	247	glu	val	gly	met	met	lys	gly	gly	ile	arg	lys	asp	arg	arg	gly
v-erbA	101	ser	val	gly	met	ala	met	asp	leu	val	leu	asp	asp	ser	lys	arg

Fig. 3. Amino acid sequence alignment of the cysteine-, lysine-, and arginine-rich region of MCF-7 human ER, human GR, and putative v-erbA oncogene product. Amino acid residues 185 to 250 from ER were aligned with residues 421 to 486 from GR and residues 37 to 104 from p75^{erbA}; common residues are boxed and gaps are indicated by dashes. Matching cysteine residues are indicated by dots above the sequence.

doubled by including $10^{-4}M$ Zn^{2+} in the culture medium for 24 hours prior to cell harvest. This result is consistent with the induction of the metallothionein promoter by zinc (18). The formation of 8–10S salt-sensitive receptor-hormone complexes in hypotonic extracts of responsive cells is a hallmark of steroid receptors, although the biological significance of these multimeric complexes has not been established. It is interesting that, although CHO-K1 cells appear to express little or no ER, the human ER expressed by OR8 cDNA in these cells forms an 8 to 9S complex when occupied by [³H]estradiol under hypotonic conditions. This suggests either that this complex is a multimer of steroid-binding subunits or that associated nonsteroid-binding components are present in nontarget cells.

Identification of the locations and properties of the functional domains on the receptor protein can help establish the mechanism by which ER regulates gene transcription. Immunohistochemical analyses of partially proteolyzed MCF-7 human ER have identified at least two regions which are separable by enzymatic cleavage: a DNA-binding region and a steroid-binding region (17). Similar analyses of rat glucocorticoid receptor (GR) revealed a third "immunogenic" domain near the amino terminus of GR (19, 20) which is probably not present in ER. When the amino acid sequences of human ER and GR (12, 20) were compared, a striking

homology was observed in a region rich in cysteine, lysine, and arginine (Fig. 3). This region occurs 300 to 350 amino acids from the carboxyl terminus in both ER and GR. Between residues 185 and 250 for ER and residues 421 and 486 for GR (66 amino acids) there are 40 matches (61 percent). A similar homology exists among ER, GR (20), and a region of the putative avian erythroblastosis virus oncogene protein p75^{erbA} (20, 21), from residue 37 to 104. All nine cysteines of ER are conserved in this region and nine out of ten GR and v-erbA cysteines are conserved. The abundance of cysteines in such a small region is unusual, and it has been suggested that this region and the associated basic amino acids may represent a DNA-binding domain of GR and v-erbA (20). Interestingly, this is the only region of significant sequence homology between human ER and GR, although both molecules contain a proline-rich region of unknown function located upstream from the region rich in cysteine, lysine, and arginine and a relatively hydrophobic region at the carboxyl terminus. On the basis of analysis of proteolytic and cDNA fragments of ER and GR (10, 17, 19) and of the nucleotide sequence of a form of GR that does not bind steroid (20), this hydrophobic region has been suggested to be the steroid-binding domain. If the primary amino acid structures of both proteins are aligned according to Fig. 3, much of the amino terminal

immunogenic region present in GR is absent in ER, suggesting that this region may be important in distinguishing the action of GR from ER.

Thus, there is a strong relationship among two steroid receptors and the erbA proto-oncogenes, indicating that they are derived from a common primordial gene. Although the mechanisms by which steroid receptors modulate transcription and by which v-erbA promotes transformation are unknown, the common feature of these molecules is a domain that may be involved in DNA recognition. Further elucidation of the role of this domain and other functional regions in the regulation of gene expression should follow from in vitro and in vivo studies of the interaction of genetically altered receptor molecules with hormone-responsive genes.

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An Ancient Developmental Induction: Heat-Shock Proteins Induced in Sporulation and Oogenesis

STEPHEN KURTZ, JANICE ROSSI, LAWRENCE PETKO, SUSAN LINDQUIST

Every eukaryotic and prokaryotic organism tested to date synthesizes a small number of heat-shock proteins in response to heat and other forms of stress. A particular pattern of heat-shock gene expression was observed during ascospore development in *Saccharomyces*: heat-shock proteins hsp26 and hsp84 were strongly induced, whereas hsp70, the most highly conserved of these proteins, was neither induced nor inducible by heat shock. Instead, two proteins related to hsp70 were induced. A strikingly similar pattern of expression occurs during oogenesis in *Drosophila*, suggesting that it may be one of the earliest developmental pathways to evolve in eukaryotic cells.

ALL ORGANISMS RESPOND TO MILD elevations in temperature by coordinately synthesizing a small set of heat-shock proteins. The exact number of proteins induced varies in different organisms, but in all cases proteins of approximately 84 and 70 kilodaltons (hsp84 and hsp70) are among the most prominent species. These proteins have been highly conserved in evolution. The hsp70 proteins of *Drosophila* and yeast have 72 percent amino acid identity (1) and their respective hsp84 proteins have 63 percent identity (2, 3). Most organisms also produce heat-shock proteins of 20 to 30 kD: *Drosophila* cells produce four closely related proteins of 28, 26, 23, and 22 kD. Cells of the yeast *Saccharomyces cerevisiae* produce only one prominent small protein with a molecular mass of 26 kD. These small heat-shock proteins have not been conserved to the same extent as hsp70 and hsp84, but nucleic acid sequence analysis has demonstrated homology among the proteins of insects, vertebrates, and nematodes (4-5). Furthermore, the small heat-shock proteins of *Drosophila*, yeast, and tomatoes form particles of highly conserved morphology (6, 7).

Although the specific functions of the heat-shock proteins are not yet known, some of them are expressed during oogenesis and pupation in *Drosophila* (8-12), suggesting that they play a role in normal development as well as in the response to stress. To investigate developmental regulation of the heat-shock genes in the yeast *S. cerevisiae*, we examined sporulating cells.

Since these cells do not efficiently take up radiolabeled amino acids, gene expression was determined with DNA probes and antibodies. In the experiment represented in Fig. 1, diploid cells of the strain AP3 reached the tetranucleate stage 8 to 10 hours after transfer to nitrogen-deficient medium and sporulation was complete at 24 hours. Total cellular RNA's were isolated at various times during sporulation, electrophoretically separated, and analyzed by hybridization with cloned probes for the heat-shock genes.

Messenger RNA (mRNA) for hsp26 was induced early in sporulation, eventually reaching a concentration higher than that achieved during a 1-hour heat shock (Fig. 1a). Messenger RNA for hsp84 was also induced during sporulation (Fig. 1b). The timing of its accumulation was different from that of the hsp26 message. The maximum level of induction was comparable to that achieved with a 1-hour heat shock.

The hsp70 gene family in *Saccharomyces* contains two different classes of heat-inducible genes encoding 70-kD proteins. Transcripts from one class, represented here by clone YG100, are observed at low levels at 25°C and at much higher levels at 36°C. Transcripts of the other, represented by clone YG107, are observed only at temperatures above 38°C (13). Neither class was induced during sporulation (Fig. 1c). Moreover, as can be seen with longer exposures, as sporulation proceeded, the concentration of the YG100 message dropped below the basal level observed during normal vegetative growth.

We examined the expression of heat-shock RNA's in several *S. cerevisiae* strains of widely divergent genotypes. Messages for hsp26 and hsp84 are induced at different times in strains that sporulate at different rates, but they are always induced strongly. Neither class of hsp70 message was induced during sporulation in any strain. Thus, unlike the coordinate induction of these genes during heat shock, their induction during development is uncoupled; only a particular subset of heat-shock genes is induced.

This pattern of heat-shock gene expression is remarkably similar to one reported to occur during normal oogenesis in *Drosophila*. In adult females, RNA's for hsp26, hsp28, and hsp84 are induced in ovarian nurse cells and passed into the developing oocyte (11). As with meiosis in *S. cerevisiae*, this developmental induction differs from heat-shock induction in that mRNA for hsp70 does not accumulate. In fact, in late egg chambers and early embryos, hsp70 is not induced even with heat shock (11). This is significant, since, in virtually all other tissues, hsp70 is the protein most strongly induced by heat.

To determine whether hsp70 is heat-inducible during sporulation, we removed portions of a sporulating culture at various times during development and subjected them to heat shock at 39°C. RNA's from these cells were hybridized with probes for the two classes of hsp70 genes. Both were inducible during the early stages of sporulation; neither was inducible during the final stages of spore maturation (Fig. 2a). This change apparently occurred before general transcriptional inactivation of the spore genome, since an mRNA encoding a 21.5-kD sporulation-specific polypeptide accumulated after hsp70 became refractory to induction (Fig. 2b).

We translated RNA's from sporulating cells in vitro and found that the heat-shock messages they contained were fully translatable. However, the fact that these RNA's can be translated in vitro provides no infor-

Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637.

Hormone-conditional transformation by fusion proteins of c-Abl and its transforming variants

Peter Jackson^{1,2,3}, David Baltimore^{2,4} and Didier Picard^{1,5}

¹Département de Biologie Cellulaire, Université de Genève, Sciences III, 30 Quai Ernest-Ansermet, CH-1211 Genève 4, Switzerland and

²Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA

³Present address: Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, CA 94143-0448, USA

⁴Present address: The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA

⁵Corresponding author

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Fusion of the hormone binding domain (HBD) of steroid receptors to transcription factors renders them hormone-dependent. We show here that an SH3-deleted, oncogenic variant of the Abl tyrosine kinase becomes hormone-dependent for transformation by fusion to the estrogen receptor (ER) HBD, extending the phenomenon to tyrosine kinases. Surprisingly, fusion of the HBD to the normal, non-transforming c-Abl (IV) protein activated transforming activity in a hormone-dependent fashion. In the presence of hormone, the c-Abl:ER fusion protein was transforming, cytoplasmic and tyrosine phosphorylated, whereas it was non-transforming, nuclear and hypophosphorylated without hormone. We have examined the kinetics of activation of the c-Abl:ER protein and found that protein synthesis is required both for kinase activation and for redistribution of the c-Abl:ER protein from the nucleus to the cytoplasm. We suggest that the activation of c-Abl could be due to HBD-mediated dimerization and/or to the ability to overexpress conditionally the normally toxic c-Abl protein. This novel approach may be applicable to a wide variety of proteins, particularly when activating mutations or physiological inducers are unknown or when the protein is toxic to cells.

Key words: Abl/conditional mutations/hormone binding domain/estrogen receptor/fibroblast transformation/tyrosine kinase

Introduction

Protein tyrosine kinases have been intensively studied for their roles in growth regulation, differentiation, and oncogenesis. For the transmembrane tyrosine kinases with identified ligands, such as the receptors for epidermal growth factor, platelet-derived growth factor, colony stimulating factor 1 and fibroblast growth factor, both the inductive signal and aspects of the mechanistic control of the kinase activity are partially understood (Yarden and Ullrich, 1988). Notably, these receptors are thought to be activated by

growth factor-induced aggregation or dimerization (Ullrich and Schlessinger, 1990). However, for the non-receptor tyrosine kinases of the Src, Abl and Fps families, the inductive signals and mechanisms are less clear, making their normal physiology difficult to study. Only recently have potential growth factor signals and regulatory mechanisms been suggested for members of the Src family, notably c-Lck, in the context of T cell signaling (Bolen and Veillette, 1989; Hatakeyama *et al.*, 1991). These non-receptor tyrosine kinases have been studied largely as transforming variants, which may only partially mirror their normal physiological roles. Clearly, the possibility of regulating manipulated variants of these kinases by exogenous signals that do not perturb normal physiology could help to assess downstream events.

Control of transforming processes dependent on non-receptor tyrosine kinases has been accomplished using temperature-sensitive mutants of transforming genes, such as *src* or *abl* (Anderson *et al.*, 1987; Engelman and Rosenberg, 1987; Kipreos *et al.*, 1987). However, for *in vivo* studies in mammalian systems, such as the investigation of Abl-induced leukemia in mice, temperature-sensitive mutations are of limited utility. In the case of transmembrane receptors, functional substitution of a heterologous extracellular domain for that normally associated with the tyrosine kinase has also been reported and may be useful for defining the role of a particular receptor without using the natural ligand (see, for example, Seedorf *et al.*, 1991; Yan *et al.*, 1991, and references therein).

Recently, regulable variants of several transcription factors have been constructed by fusion to the hormone binding domains (HBDs) of the glucocorticoid receptor or the estrogen receptor (ER). These regulatory domains can inactivate the heterologous activity in a fashion that can be reversed by hormone (Picard *et al.*, 1988; Eilers *et al.*, 1989; Burk and Klempnauer, 1991; Superti-Furga *et al.*, 1991; Umek *et al.*, 1991). It has been proposed that the inactivation is mediated by the hormone-reversible association of the HBD with a heat shock protein, HSP90 (Picard *et al.*, 1988). We now report that this strategy can also be applied to a tyrosine kinase. Thus, a transforming derivative of *c-abl* becomes a hormone-inducible oncogene by gene fusion to a region encoding a steroid binding domain. Surprisingly, we discovered that a normal, non-transforming c-Abl could be activated by fusion to the HBD of the ER. In the presence of hormone, this hybrid protein becomes a potent transforming protein despite being composed of two oncogenically inactive moieties. We use this hormone-regulable Abl kinase to study the kinetics of kinase activation, to identify a protein synthesis requirement for kinase activation, to correlate the formation of a high molecular weight complex with Abl-dependent transformation and to show transformation-dependent redistribution of the Abl protein from the nucleus to the cytoplasm.

Results

Construction of hormone-regulable derivatives of the Abl kinase

To construct hormone-regulable mutants of the Abl protein, we examined whether the regulation of transforming activity by the ER HBD observed for transcription factors could be extended to a transforming variant of Abl.

Hormone-reversible inactivation of transforming Abl by fusion to a steroid receptor hormone-binding domain. To express a hormone-inducible derivative of Abl, we constructed a retroviral vector, pPL, encoding a fusion protein, denoted by Δ XB:ER, including the N-terminal domains of the Δ XB transforming mutant of c-Abl (IV) fused to the human ER HBD (Figure 1). The original Δ XB mutant had a deletion of the Src homology domain 3 (SH3) of the

non-transforming c-Abl (IV) and possessed the same transforming properties as v-Abl (Jackson and Baltimore, 1989). The C-terminal domain of Abl has several other potential functions including DNA binding, actin association and a role in some transformation assays, but is not required for fibroblast transformation (Prywes *et al.*, 1985; Van Etten *et al.*, 1989; Kipreos and Wang, 1992). Thus, to simplify our analysis, the domain C-terminal to the major nuclear localization signal was deleted in the Δ XB:ER fusion protein. As a control for this deletion, we constructed a similar C-terminal deletion mutant of Δ XB without fusion to the HBD, Δ XB- Δ 630 (Figure 1). Retroviral vectors encoding these mutants and the parental c-Abl and Δ XB mutants were cotransfected with a cloned Moloney helper virus into NIH 3T3 cells, which were plated with and without estradiol.

The Δ XB:ER and Δ XB- Δ 630 constructs produced similar

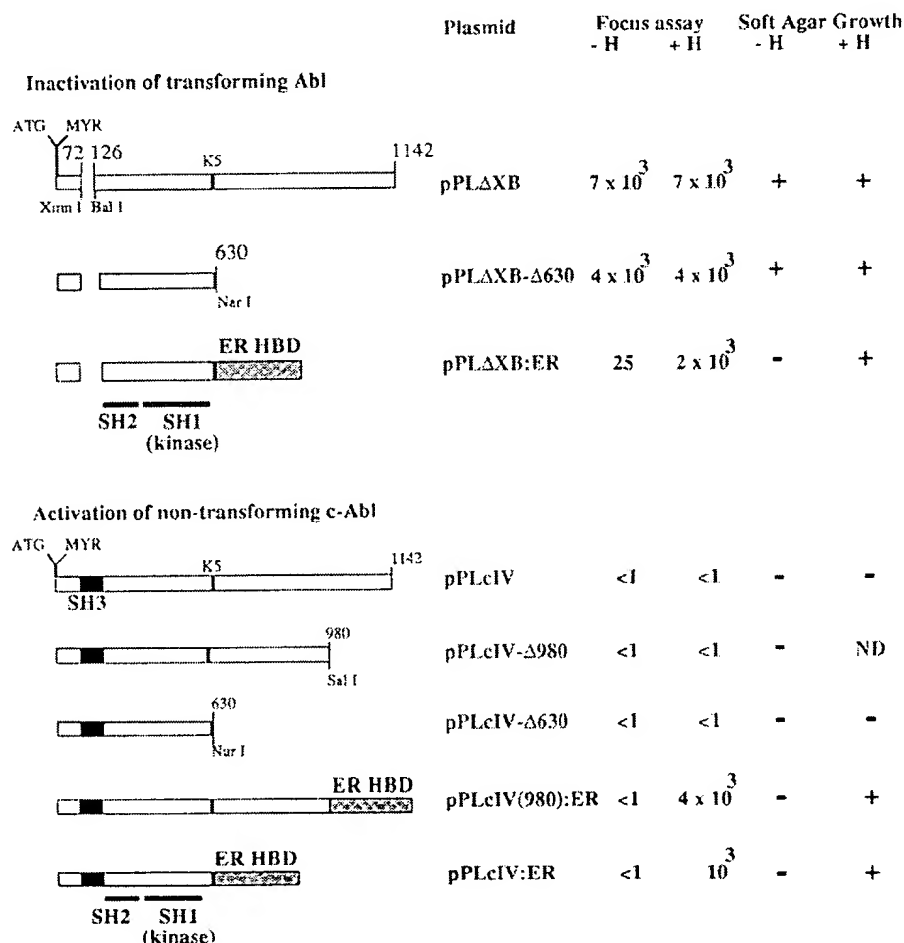


Fig. 1. Schematic representations and biological properties of Abl protein derivatives fused to the ER HBD. c-Abl sequences and ER HBD are represented by open and shaded boxes, respectively. Several c-Abl characteristics are indicated: ATG, initiator codon; MYR, myristoylation site; SH1, SH2 and SH3, Src homology domains 1, 2 and 3, respectively; K5, the pentalysine motif of the c-Abl nuclear localization signal. Landmark restriction sites used for deletion are indicated. Note that transforming Abl variants lack SH3. (Above) Dominant inactivation of transforming Abl. pPL Δ XB: an SH3 deletion mutant of c-Abl (IV) (Jackson and Baltimore, 1989). pPL Δ XB- Δ 630: a double mutant of c-Abl (IV) with both the SH3 deletion and the C-terminal deletion of residues 631–1142. pPL Δ XB:ER: a fusion protein with the SH3 deletion mutant of c-Abl (IV), Δ XB- Δ 630, and residues 282–595 of the human ER. (Below) Activation of normal, non-transforming c-Abl. pPLcIV: a retroviral vector encoding full-length c-Abl (IV) (Jackson and Baltimore, 1989). pPLcIV- Δ 980: a derivative of pPLcIV, encoding c-Abl (IV) with C-terminal residues 981–1142 deleted. pPLcIV- Δ 630: a derivative of pPLcIV, encoding c-Abl (IV) with C-terminal residues 631–1142 deleted. pPLcIV(980):ER: a fusion protein containing residues 1–980 of c-Abl (IV) and residues 282–595 of the human ER. pPLcIV:ER: a fusion protein containing residues 1–630 of c-Abl (IV) and residues 282–595 of the human ER. Constructs were cotransfected with a cloned M-MuLV provirus (pZAP) into NIH 3T3 cells. On day 2, the transfected cells were split into media with or without 1μ M estradiol (+H or -H). Transformed foci were counted on day 10 post-transfection. 'Transformation' refers to the number of morphologically transformed foci per plate (normalized for the dilution when split). Populations of transfected cells were plated in 0.48% Difco agar in DME with 10% donor calf serum either with or without 1μ M β -estradiol. 'Soft agar growth' was scored by counting macroscopic colonies at 21 days.

numbers of morphologically transformed foci in the presence of hormone, but the Δ XB:ER construct produced ~80-fold fewer foci in the absence of hormone. The foci formed in the absence of hormone were small and could not be passaged as cell lines without the addition of hormone. This failure of lines to grow without hormone is probably due to a strong cytostatic effect of the Abl:ER fusion protein in the absence of hormone (P.Jackson, D.Baltimore and D.Picard, in preparation). Further, the cells transformed by the Δ XB:ER construct failed to grow without hormone in the more stringent soft agar growth assay, but did form colonies in agar in the presence of estradiol (Figure 1). The Δ XB- Δ 630 mutant transformed cells only slightly less well than the parental Δ XB mutant, and these mutants were not affected by hormone in either the focus formation or soft agar growth assays (Figure 1). Thus, the ER HBD inactivated the transforming ability of the Δ XB mutant in a hormone-reversible fashion. Similar results were obtained with the HBD of the glucocorticoid receptor (data not shown).

Oncogenic activation of non-transforming c-Abl by fusion to the ER hormone binding domain. Initially intended as a negative control, we constructed a vector encoding a c-Abl:ER hybrid protein retaining the SH3 domain, called cIV:ER (Figure 1). We were surprised to find that this

construct also transformed NIH 3T3 cells. While no foci appeared in the absence of hormone, the cIV:ER construct transformed quantitatively almost as well as the Δ XB:ER or Δ XB- Δ 630 constructs in the presence of hormone (Figure 1). As expected, the C-terminal deletion mutant cIV- Δ 630 did not transform with or without estradiol, demonstrating that the simple deletion of the C-terminus did not activate the transforming ability of c-Abl (IV). Thus, the HBD of the human ER activated c-Abl (IV) in the presence of hormone.

A different c-Abl:ER hybrid protein including more of the Abl C-terminus, cIV(980):ER (Figure 1) was also activated for transformation in the presence of hormone. Thus, the ER HBD will function in different contexts within the c-Abl protein to activate transforming ability.

Abl:ER fusion proteins with and without SH3 transform reversibly, but with different kinetics

We examined whether the transforming effect of the Abl:ER fusion proteins was reversible in stable, clonal cell lines. Populations of NIH 3T3 cells transformed by the cIV:ER and Δ XB:ER mutants were grown in estradiol and cloned by limiting dilution. In multiple clonal NIH 3T3 lines expressing the cIV:ER fusion protein, cells grown in the

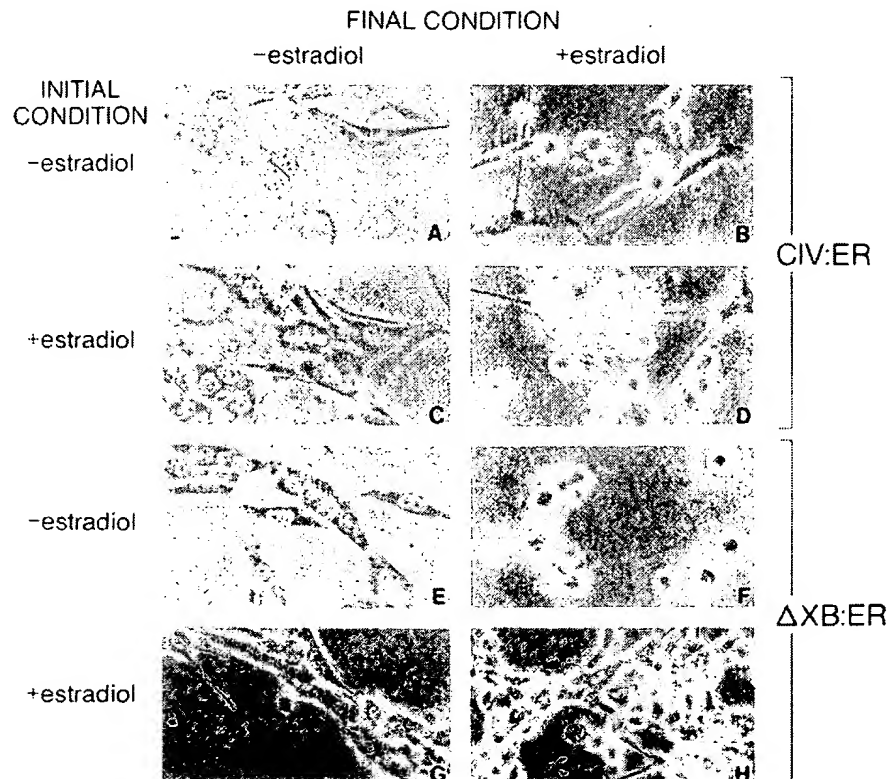


Fig. 2. Hormone-dependent morphological transformation. Clonal lines expressing the p103^{cIV:ER} and p96 ^{Δ XB:ER} fusion proteins were grown in the presence of estradiol (1 μ M) in a morphologically transformed state. Cells were washed and plated either with or without estradiol and grown for 2 days (INITIAL CONDITION). Plates were then washed and fed with or without estradiol, and grown for an additional 2 days (FINAL CONDITION). (A–D) NIH 3T3 cells expressing p103^{cIV:ER}. (E–H) NIH 3T3 cells expressing p96 ^{Δ XB:ER}. (A and E) Cells grown for 2 days without estradiol reverted fully and maintained their flat morphology. (B and F) Cells were grown for 2 days without estradiol and reverted fully. These cells were treated with estradiol and grown for an additional 2 days. Much of the fully transformed morphology was seen at day one. (C and G) Cells in estradiol showing a fully transformed morphology were washed and refed with medium lacking hormone and grown for an additional 2 days. The reversion is complete with the cIV:ER cells, while the reversion of the Δ XB:ER cells is substantial, but slower and more variable. (D and H) Cells that have been grown for 4 days in estradiol maintain their transformed morphology and grow considerably faster than control cells without hormone. Scale: Nuclear diameter in photomicrograph A is ~10 μ m.

presence of estradiol were highly morphologically transformed. When viewed by phase contrast microscopy, the cIV:ER cells were homogeneously round and refractile, grew to high density without apparent contact inhibition, and, when viewed by time-lapse video microscopy, were highly motile with rapidly changing, extended cellular processes and reduced lamellipodia. In the absence of hormone, the cIV:ER cells were uniformly flat, showed normal, non-refractile cytoplasmic and phase-dark nuclear structure, low motility and ruffling lamellipodia. In the absence of hormone, the ΔXB:ER cells behaved similarly, but were less homogeneously flat, with some cells showing intermediate morphologies (data not shown).

When clonal lines that had been transformed in the presence of hormone by the cIV:ER mutant were washed and plated in the absence of hormone (3600 cells/cm²), most of the cells showed a flat morphology within 1 day. By the second day, the cells had homogeneously reverted (Figure 2). The rate of reversion was somewhat lower at high cell density (>26 000 cells/cm²). Adding hormone to cells that had reverted induced their morphological transformation (Figure 2). During the time course following hormone addition, intermediate transformed morphologies were recognizable, but within 2 days the cells were morphologically indistinguishable from cells grown continuously in hormone or from NIH 3T3 cells transformed by v-Abl (Figure 2 and data not shown). Thus, the transformed phenotype was reversibly dependent on the continued presence of estradiol.

Cells transformed by the ΔXB:ER fusion protein required more time to revert homogeneously after removal of hormone than the cIV:ER expressing cells, but nonetheless showed substantial reversion within 2 days. Reverted cells were induced to a transformed phenotype by addition of hormone within 1 day (Figure 2). Cells with the ΔXB:ER fusion protein apparently reverted with more difficulty and were induced more easily than cells with the cIV:ER fusion protein, suggesting that the ΔXB:ER fusion protein had higher levels of transforming activity in cells. Growth of the cIV:ER expressing lines in soft agar was also somewhat slower than that of the ΔXB:ER-expressing lines in the presence of hormone. Thus, the transforming activities of both the ΔXB:ER and cIV:ER fusion proteins were hormone-dependent and reversible, but with different kinetics of morphological response to hormone.

Abl:ER fusion proteins with and without SH3 have different hormone dosage responses for hormone-induced transformation

Because the cIV:ER- and ΔXB:ER-expressing lines had different kinetic responses to hormone, we examined the dose-dependence for growth in soft agar. The ΔXB:ER fusion protein responds half-maximally to hormone at concentrations slightly higher than the known K_d of the HBD for estradiol (see Table I) and consistent with the dosage response of a myc:ER fusion protein (Eilers *et al.*, 1989). In contrast, half-maximal stimulation of the cIV:ER fusion protein required ~20-fold more hormone (Table I). Both showed a sigmoidal response to varying concentrations of hormone (data not shown). Thus, there were differences in hormone responsiveness of the cells expressing the Abl:ER fusion proteins with or without the SH3 domain.

To address the possibility that the Abl:ER fusion proteins

Table I. Hormone binding and induction of Abl:ER fusion proteins

Cell line	Number of molecules per cell	Hormone-binding affinity (K_d) ^a	Half-maximal dose for growth in soft agar ^b (nM)
ΔXB:ER	$4.2 \times 10^6 \pm 24\%$	$2.5 \text{ nM} \pm 18\%$	~8
cIV:ER	$2.0 \times 10^6 \pm 14\%$ ^c	$13.3 \text{ nM} \pm 24\%$	~150

^aAffinity for β-estradiol.

^bEstimated by graphical analysis of the β-estradiol dose-response curve for growth in soft agar.

^cThis is a minimum estimate because these cells were less adherent in serum-free medium and some cells were unavoidably lost during washing. Loss of cells during washing would not affect the measured affinity, but might cause the number of binding sites per cell to be underestimated.

with or without SH3 had different affinities for hormone, we performed *in vivo* hormone binding studies. Cells expressing the cIV:ER fusion protein bound [³H]estradiol with ~5-fold lower affinity than the ΔXB:ER cells (Table I). Both fusion proteins expressed several million hormone binding sites per cell, consistent with high levels of Abl:ER fusion protein expression (>10-fold more than endogenous c-Abl). Although the cIV:ER fusion protein has a lower affinity for ligand than the ΔXB:ER fusion protein, this difference cannot account for the 20-fold difference in hormone level required for transformation. Possibly, the difference in behavior of the two fusion proteins could be due to an inhibitory function of SH3 in c-Abl (Franz *et al.*, 1989; Jackson and Baltimore, 1989; Pendergast *et al.*, 1991; Cicchetti *et al.*, 1992) or to differences in subcellular localization of the two fusion proteins (see below).

The transforming activities of the Abl:ER fusion proteins correlate with hyperphosphorylation on tyrosine

A characteristic biochemical difference between *abl*-transformed cells and parental cells is the presence of specific proteins phosphorylated on tyrosine residues, including Abl itself. We were interested in whether induction of tyrosine phosphorylation of these proteins occurred in cells expressing the Abl:ER fusion proteins after hormone addition. Probing an immunoblot for Abl protein showed that addition of hormone to reverted cells caused a slight reduction in the electrophoretic mobility for the 103 kDa cIV:ER fusion protein (Figure 3). An immunoblot performed in parallel using an anti-phosphotyrosine antibody showed that the less mobile form of the cIV:ER doublet seen in the presence of hormone is tyrosine phosphorylated, while in the absence of hormone, the more mobile form did not appear to be tyrosine phosphorylated (compare panels in Figure 3). Similarly, in cells expressing the 96 kDa ΔXB:ER fusion protein in the presence of hormone, this protein appeared to be hyperphosphorylated. In the absence of hormone, the ΔXB:ER protein appeared significantly less tyrosine phosphorylated (~5-fold) but did maintain some basal tyrosine phosphorylation. In some experiments, a subtle size shift dependent on hormone was apparent for the ΔXB:ER protein also (data not shown). Thus, the transforming forms of the Abl:ER fusion proteins had higher levels of tyrosine phosphorylation than the non-transforming forms.

We also examined the *in vitro* kinase activity of the Abl:ER

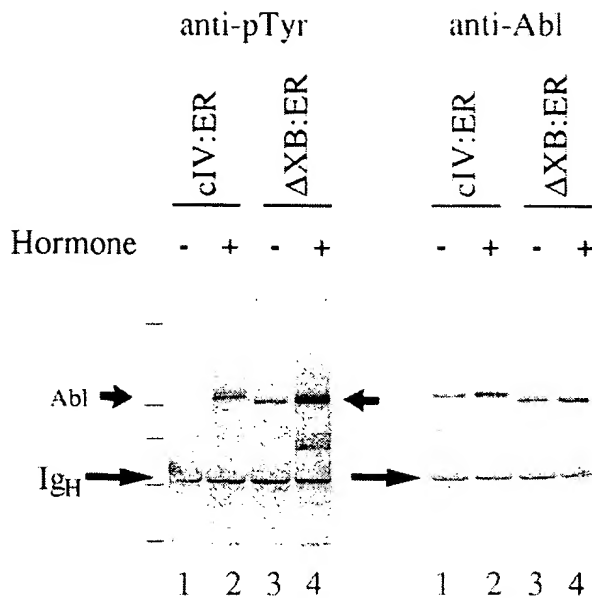


Fig. 3. Hormone-dependent induction of Abl-dependent phosphorylation. Clonal lines expressing the cIV:ER and Δ XB:ER fusion proteins were washed, plated without estradiol and grown for 2 days to allow the cells to revert. Plates of reverted cells were treated with hormone or mock treated for 2 days. Extracts were immunoprecipitated with antiserum specific for c-Abl (IV), run on 5–15 % SDS–polyacrylamide gradient gels, blotted on to Immobilon (Millipore), and stained for anti-phosphotyrosine or Abl determinants (see Materials and methods). Lanes 1, cIV:ER cells without hormone; lanes 2, cIV:ER cells with 1 μ M estradiol; lanes 3, Δ XB:ER cells without hormone; lanes 4, Δ XB:ER cells with 1 μ M estradiol. **Left panel:** anti-phosphotyrosine Western blot shows an increase in the level of Abl tyrosine phosphorylation (compare with right panel for normalization) from low basal levels to high levels for cIV:ER-expressing cells and from moderate basal levels to high levels for Δ XB:ER-expressing cells. **Right panel:** anti-Abl Western blot. Note the comigration of the upper species with the hyperphosphorylated form of Abl for cIV:ER. Abl species are indicated with short arrows and the heavy chain of the precipitating antibody with a long arrow. Molecular weight markers on the left are 30, 46, 69, 97.4 and 200 kDa.

proteins in an immune complex kinase assay and found no apparent hormonal effect. Both cIV:ER and Δ XB:ER proteins had levels of kinase activity proportional to the amount of protein immunoprecipitated from cells treated either with or without hormone (not shown) suggesting that the *in vitro* kinase assay did not reflect the *in vivo* activity. Under the conditions of the assay, interaction with HSP90, which might be required for any hormone-dependent regulation, would not be maintained. Direct addition of hormone to the immune complex kinase assay also had no apparent effect (not shown).

The SH3 domain delays the induction of tyrosine phosphorylation

We examined the kinetics of activation of phosphorylation of cellular proteins by anti-phosphotyrosine Western analysis. Clonal lines expressing the cIV:ER and Δ XB:ER fusion proteins were plated in the absence of hormone and allowed to revert for 2 days. Estradiol was added to 1 μ M ($t = 0$) and lysates were prepared at various times and examined by anti-Abl and anti-phosphotyrosine Western analysis.

The cIV:ER mutant showed a subtle increase in Abl protein level over 24 h, but a more dramatic increase in autophosphorylation within 1–2 h and then transphosphorylation of previously noted cellular proteins within one to several hours (Figure 4A and C). These tyrosine phosphorylated species are identical to those seen in NIH 3T3 cells transformed by v-Abl or an SH3-deleted c-Abl (species indicated are 36, 62, 85, 210/220 and 280–300 kDa). The general kinetics of activation of the cIV:ER fusion protein was reproducible but showed some variation in the time required to show strong anti-phosphotyrosine staining (2–6 h).

The Δ XB:ER mutant showed a different kinetics of activation. Both the autophosphorylation/size shift of the Δ XB:ER Abl species and the transphosphorylation of the cellular species occur in < 10 min (Figure 4B and D). In contrast, an increase in the level of the Δ XB:ER protein requires > 10 h. Thus, the activation of the Δ XB:ER protein by hormone addition occurs with faster kinetics than that for the activation of the cIV:ER protein.

The background level of tyrosine phosphorylated species in the Δ XB:ER expressing cells was higher in the absence of hormone than for the cIV:ER cells, suggesting that the Δ XB:ER protein has some kinase activity without hormone. However, phosphorylation of these background species is not induced by hormone, suggesting that they are not relevant substrates for transformation. In contrast, the phosphorylation of some species like the 62 kDa protein is highly dependent on hormone (Figure 4D), suggesting that these may be crucial substrates for transformation.

Protein synthesis is required for activation of cIV:ER, but not Δ XB:ER

When reverted cells were pretreated with cycloheximide for 30 min before addition of hormone, the level of the p103^{cIV:ER} protein decreased only slowly over the 18 h time course as seen by Western blotting (Figure 5A). Inspection of an anti-phosphotyrosine Western blot showed no activation of phosphotyrosine-containing proteins by the Abl kinase in the presence of cycloheximide (Figure 5C).

Cells expressing the SH3-deleted Abl:ER fusion protein, p96 ^{Δ XB:ER}, did show considerable induction of tyrosine phosphorylation of characteristic species, even in the presence of cycloheximide (Figure 5D). Furthermore, the level of phosphotyrosine in these proteins was maintained in the absence of protein synthesis for at least several hours (Figure 5B and D). This suggests that cycloheximide blocks activation of the tyrosine kinase activity of the existing pool of cIV:ER fusion protein, and rules out a failure to maintain tyrosine phosphorylated proteins in the absence of protein synthesis. This requirement for protein synthesis could reflect the need to synthesize either new cIV:ER protein or proteins required for Abl activation, or both. Cycloheximide apparently inhibited the ability to induce morphological transformation for both cIV:ER and Δ XB:ER lines, but the toxicity of the drug on this time scale made this experiment difficult to interpret.

Immunofluorescence shows hormone-induced redistribution of cIV:ER from nucleus to cytoplasm

Our previous work suggested a difference in subcellular localization between normal c-Abl (IV) [which was partly nuclear and partly associated with actin-rich stress filaments

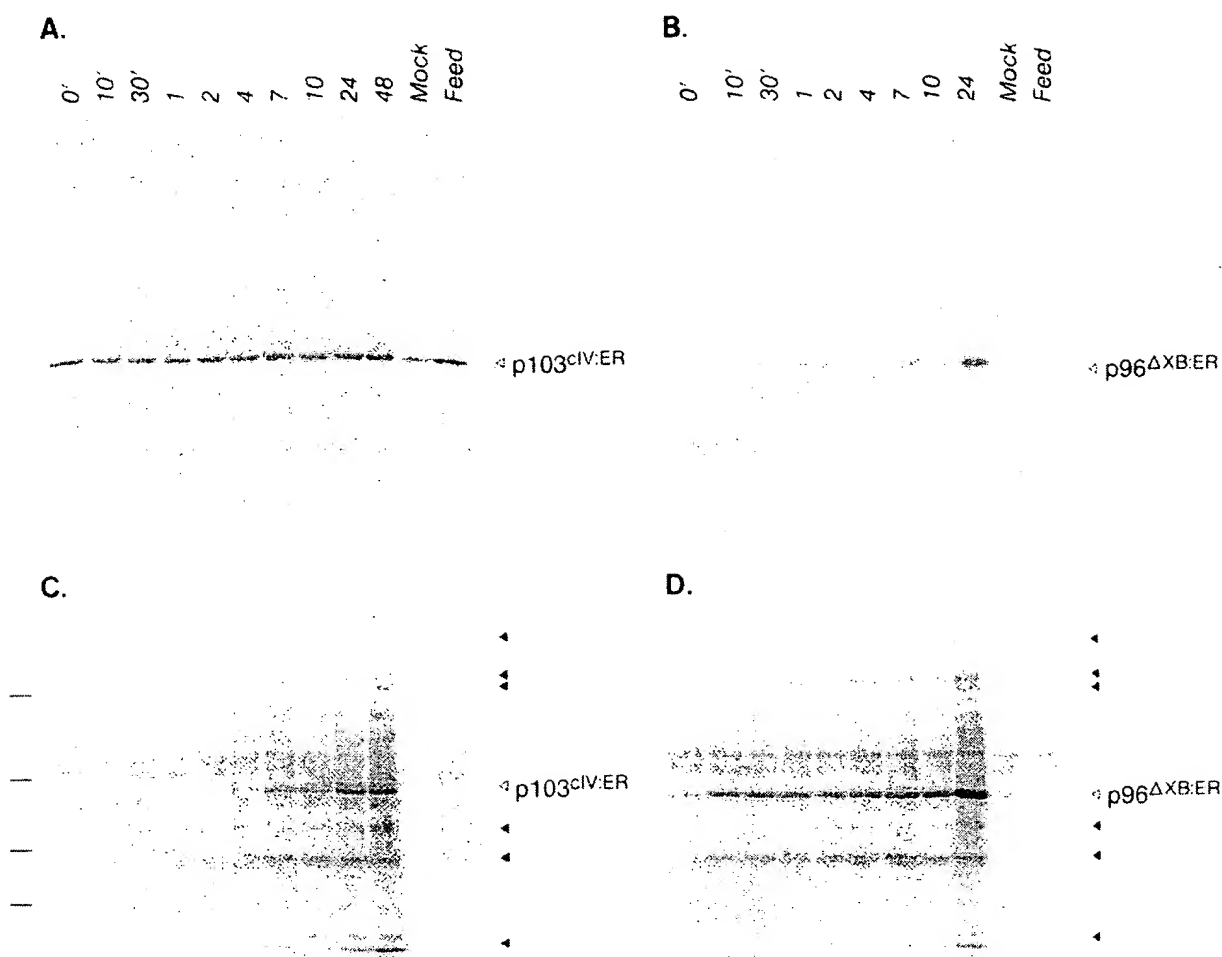


Fig. 4. Kinetics of estrogen-induced tyrosine phosphorylation. Clonal lines expressing the cIV:ER and Δ XB:ER fusion proteins were washed, plated without estradiol and grown for 2 days in order to revert the cells morphologically. Multiple plates of reverted cells were treated with hormone and extracts prepared at the indicated times. The lane labeled 'mock' indicates an equivalent plate with no treatment and the lane labeled 'feed' was refed with fresh medium without hormone. Extracts were run on 5–15% SDS–polyacrylamide gradient gels and analyzed by anti-Abl and anti-phosphotyrosine Western blotting as described in Materials and methods. (A and C) Time course of estrogen induction of cIV:ER. (B and D) Time course of estrogen induction of Δ XB:ER. (A and B) Anti-Abl Western analysis shows the p103 and p96 Abl species using a monoclonal antibody (mAb 19-84) against the kinase domain of Abl. (C and D) Anti-phosphotyrosine Western analysis shows the induction of a series of tyrosine phosphorylated species characteristic of Abl transformed cells. Six prominent induced species are 36, 62, 85, 210/220 and 300 kDa (indicated by closed triangles, starting from the bottom), which are identical to those seen in other Abl-transformed cells. The autophosphorylated Abl species is indicated by an open triangle. The induction of autophosphorylation and exogenous phosphorylation by the cIV:ER fusion protein requires at least 1 h for a detectable increase and thereafter increases cumulatively, whereas the induction of phosphorylation of these species occurs to near maximal levels within minutes for the Δ XB:ER fusion protein. The appearance of the 210 kDa band was variable. Molecular weight markers are 46.5, 77, 116.5 and 205 kDa.

(absent in transformed cells)] and transforming versions of the Abl protein, which are predominantly cytoplasmic (Van Etten *et al.*, 1989). To examine whether the localizations of the Abl:ER fusion proteins change upon activation, we analyzed these fusion proteins by indirect immunofluorescence using antisera directed either against the Abl SH3 domain or against the ER HBD.

The cIV:ER fusion protein was observed to be mostly nuclear in cells plated in the absence of hormone and allowed to revert as judged by indirect immunofluorescence staining with affinity-purified antibodies against the Abl SH3 domain (Figure 6A). The majority of staining was due to determinants from the highly overexpressed cIV:ER fusion protein and little staining from endogenous c-Abl as

suggested by the much lower level staining of parental NIH 3T3 cells (Figure 6B). A similar pattern of nuclear staining was seen with serum directed against the ER HBD, which showed low background staining of NIH 3T3 cells (Figure 6C and D). We saw no actin association, as would be expected from the fact that the cIV:ER fusion protein lacks the C-terminal actin binding domain.

We examined the localization of the cIV:ER fusion protein at various times after hormone addition and found that over 2–24 h the protein appeared to have a strong nuclear component of anti-Abl SH3 staining, but significant cytoplasmic staining became evident. A representative micrograph showing cIV:ER cells 8 h after hormone addition shows predominantly nuclear staining in some cells and very

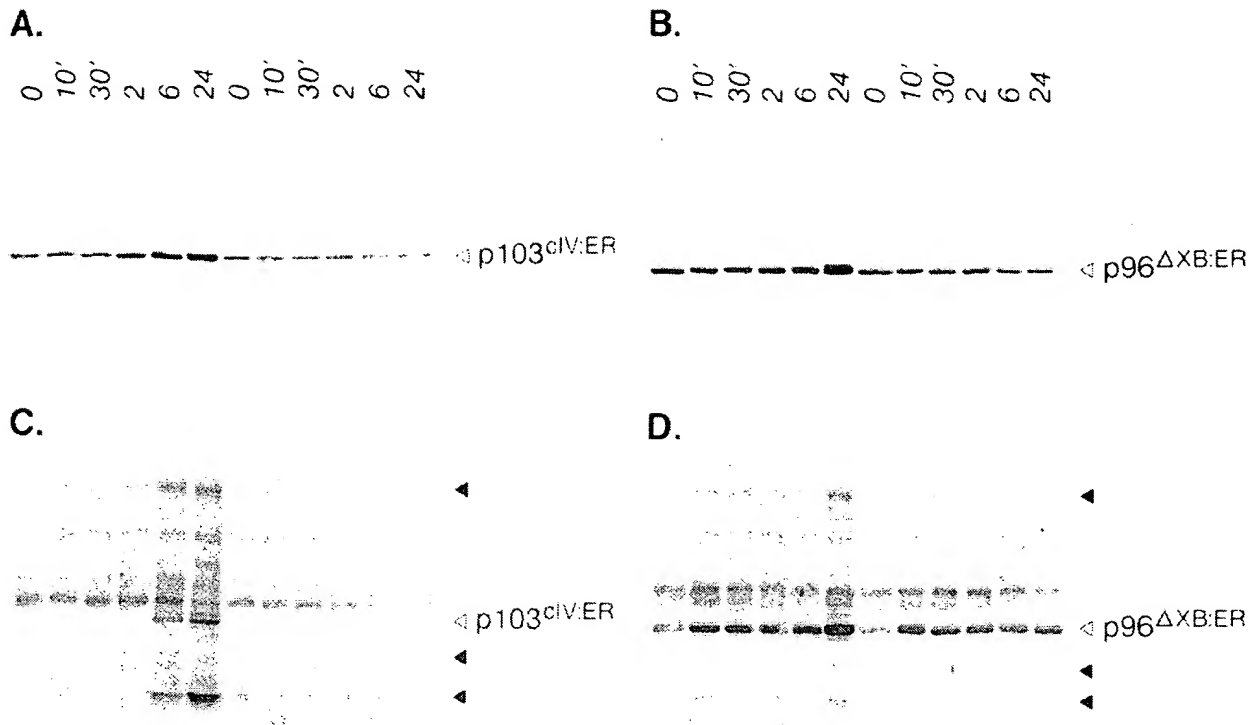


Fig. 5. Activation of the cIV:ER fusion protein kinase requires protein synthesis, whereas the SH3-deleted variant apparently loses this requirement. Clonal lines expressing the cIV:ER and Δ XB:ER fusion proteins were washed, plated without estradiol and grown for 2 days to allow the cells to revert. Multiple plates of reverted cells were treated with hormone and with or without cycloheximide at 100 μ g/ml. Extracts were prepared in the presence of the phosphatase inhibitor sodium orthovanadate at the indicated times. Extracts were run on 10% SDS-polyacrylamide gels and analyzed by anti-Abl and anti-phosphotyrosine Western blotting. The four panels are similar to those in Figure 4. In each case, the time course on the left is performed without cycloheximide, and that on the right with cycloheximide. The three closed triangles in panels C and D indicate 62, 85 and 220 kDa proteins; the open triangles indicate the Abl protein.

strong overall staining in others (Figure 6E). In general, the intensity of both nuclear and cytoplasmic staining increased during this time frame. However, between 24 and 48 h after hormone addition, as well as in cells grown continuously in hormone, much of the nuclear staining was lost in most cells, and predominantly cytoplasmic staining was seen (Figure 6F). In cIV:ER cells blocked with cycloheximide and treated with hormone, the protein appeared to remain nuclear, consistent with the cytoplasmic accumulation being due to *de novo* synthesis (data not shown). Thus, the redistribution of the cIV:ER protein concurrent with activation recapitulated the previously reported difference in subcellular localization of c-Abl and transforming versions of Abl (Van Etten *et al.*, 1989; Dhut *et al.*, 1991). However, the kinetics of the redistribution process did not suggest that the protein physically shuttled from the nucleus to the cytoplasm. Rather, the redistribution appeared consistent with a gradual cytoplasmic accumulation of *de novo* synthesized Abl because of an increasing inability of this protein to enter the nucleus in the cells undergoing transformation.

The Δ XB:ER fusion protein remains cytoplasmic

Cells expressing the Δ XB:ER fusion protein showed a distinctive perinuclear staining pattern in the absence of hormone (Figure 6K). Such a subcellular distribution has not been observed for any previously described Abl variant. A kinetic analysis of subcellular localization showed that the Δ XB:ER protein becomes diffusely cytoplasmic lacking the

strong perinuclear staining by 24 h after hormone addition (Figure 6L). Throughout the time course, there was no appreciable nuclear staining (data not shown).

Hormone-dependent induction of membrane-associated phosphotyrosine staining

We examined the subcellular localization of the accumulating phosphotyrosine-containing proteins by indirect immunofluorescence. In the absence of hormone, the cells expressing the cIV:ER fusion showed anti-phosphotyrosine antibody staining of punctate and wedge-shaped structures on the ventral surface of the cell (Figure 6G). A similar pattern is seen in parental NIH 3T3 cells and the staining of these structures is blocked with free phosphotyrosine (data not shown). These phosphotyrosine-containing structures have previously been identified as closely associated with focal adhesions and integrin receptors (Tapley *et al.*, 1989; Guan *et al.*, 1991). As early as 2 h after hormone addition, novel focal structures near the ruffling edge of the cell (Figure 6H) and actin-rich cellular processes extending from the plasma membrane (Figure 6I) frequently stain with anti-phosphotyrosine antibody in many cells. Similar membrane structures stain with the anti-Abl SH3 antibodies consistent with the c-Abl:ER fusion being one of the major tyrosine phosphorylated species (data not shown). Highly transformed cells are strongly stained with anti-phosphotyrosine antibodies at 24–48 h after hormone addition (Figure 6J), or in cells maintained in hormone continuously (not shown). A variety of other cytoplasmic structures and a punctate

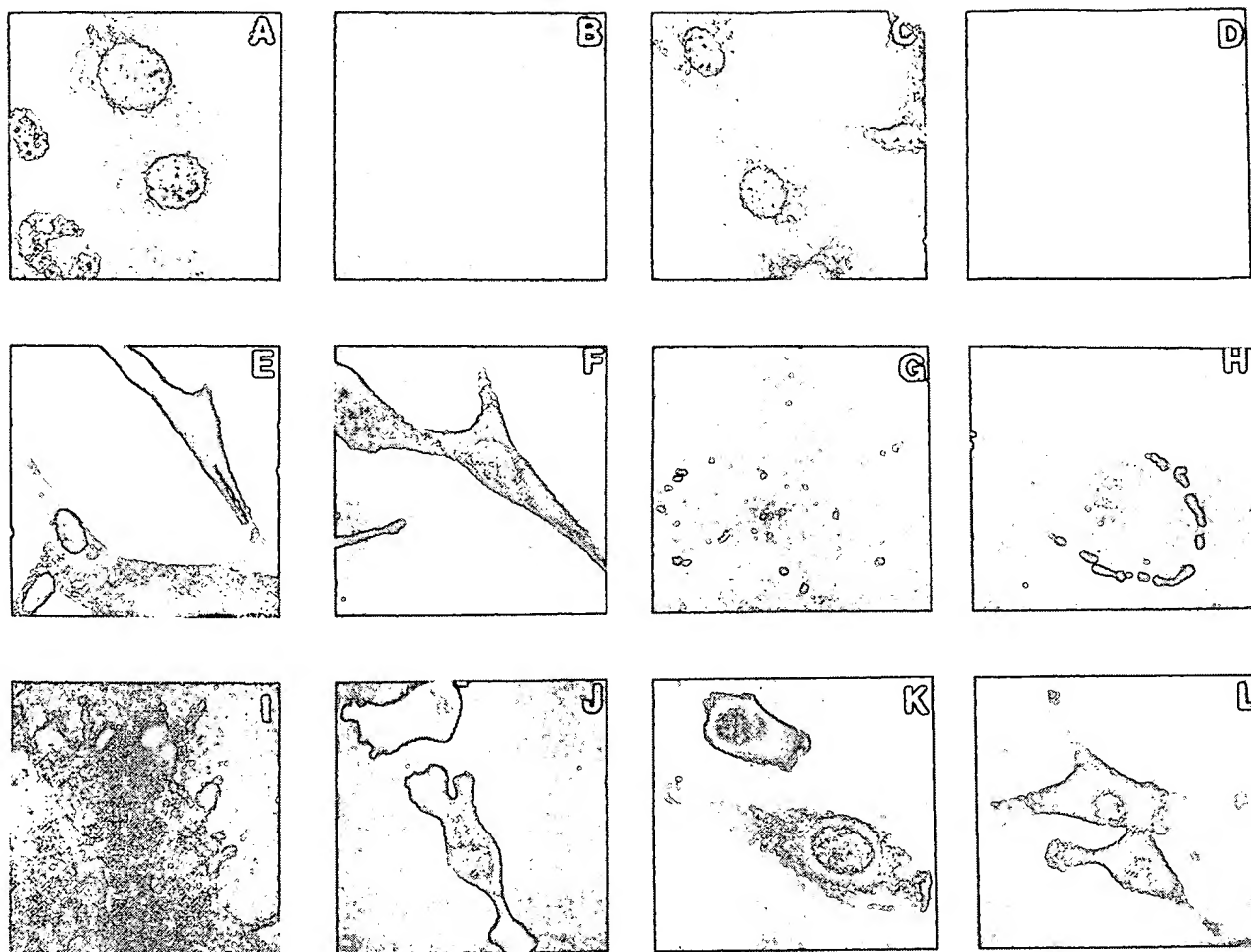


Fig. 6. Hormone-dependent activation of the cIV:ER fusion protein results in a change of localization from nucleus to cytoplasm, while the SH3-deleted variant never localizes to the nucleus. NIH 3T3 lines expressing the cIV:ER and Δ XB:ER fusions were plated in the absence of hormone and allowed to revert for 2 days. The cells were then treated with hormone and fixed for indirect immunofluorescence using methanol-acetone fixation as described in Materials and methods. Primary antibodies were either affinity-purified rabbit antiserum directed against the Abl SH3 domain (A, B, E and F), or rabbit antisera directed against the human ER HBD (C, D, K and L) or the mouse monoclonal antibody 4G10 against phosphotyrosine (G, H, I and J). Secondary antibodies were rhodamine-conjugated donkey anti-rabbit (A-D, K and L) or anti-mouse (G-J). (A) cIV:ER-expressing NIH 3T3 cells in the absence of hormone stained with SH3-specific primary serum (800 \times magnification) showing strong nuclear localization of the cIV:ER fusion protein. (B) Parental NIH 3T3 cells stained with SH3-specific primary sera showing low background. (C) cIV:ER-expressing NIH 3T3 cells in the absence of hormone stained with ER HBD-specific primary serum confirming the nuclear staining in A. (D) Parental NIH 3T3 cells stained with ER HBD-specific primary serum showing low background. (E) cIV:ER-expressing NIH 3T3 cells 8 h after hormone addition stained with SH3-specific primary serum (500 \times) retain nuclear staining, but accumulate cytoplasmic staining. (F) cIV:ER-expressing NIH 3T3 cells 48 h after hormone addition stained with SH3-specific primary serum (625 \times) showing largely cytoplasmic localization. (G) cIV:ER-expressing NIH 3T3 cells in the absence of hormone, stained with an anti-phosphotyrosine primary antibody. The focus is on the ventral surface of the cell to emphasize the staining of focal adhesions (500 \times). (H and I) cIV:ER-expressing NIH 3T3 cells 8 h after the addition of hormone, stained with an anti-phosphotyrosine primary antibody showing the appearance of membrane phosphotyrosine staining. (H) The focus is on the ruffling edge of the cell (800 \times). (I) A detailed view showing anti-phosphotyrosine staining in ruffles and spikes (158 \times). (J) cIV:ER-expressing NIH 3T3 cells 48 h after hormone addition stained with an anti-phosphotyrosine primary antibody (625 \times) showing a large accumulation of tyrosine-phosphorylated proteins. (K) Δ XB:ER-expressing NIH 3T3 cells in the absence of hormone stained with ER HBD-specific primary serum (800 \times magnification) showing a strong perinuclear localization. (L) Δ XB:ER-expressing NIH 3T3 cells 48 h after addition of hormone, stained with ER HBD-specific primary serum showing diffuse cytoplasmic localization.

nuclear structure stain with anti-phosphotyrosine antibodies at different times after hormone addition (data not shown). These may represent intermediates in the process of morphological transformation, but a more systematic analysis will be required to establish this idea. Results with Δ XB:ER were essentially similar (data not shown).

Discussion

We have developed a new strategy for constructing regulable derivatives of both normal and activated Abl tyrosine kinases

by fusing them to the ER HBD. We have exploited these hormone-conditional Abl:ER fusion proteins to study the kinetics of activation of the Abl kinase activity, the Abl-dependent induction of the transformed state and its influence on redistribution of the Abl protein from the nucleus to the cytoplasm, and to identify a protein synthesis requirement for activation of the Abl kinase.

Fusion to steroid binding domains has previously been used to construct regulable derivatives of several transcription factors. In those cases, the heterologous activity becomes inactivated in a hormone-reversible fashion (Picard

et al., 1988; Eilers *et al.*, 1989; Burk and Klempnauer, 1991; Superti-Furga *et al.*, 1991; Umek *et al.*, 1991). We have now extended the list of the regulable activities to a tyrosine kinase, a transforming variant of c-Abl. Completely unexpected was the finding that a non-transforming, normal c-Abl derivative could be activated for transformation by fusion to the ER HBD. This finding has implications both technically and for the understanding of Abl activation (see below). The activation of c-Abl is also unusual because C-terminal mutations or fusions have not generally been found to activate c-Abl. Further, activation by the ER HBD is relatively specific because C-terminal fusion of other proteins including Gag, Bcr and the ecdysone receptor HBD did not activate c-Abl (P.Jackson and D.Picard, unpublished data).

Hormone-dependent transformation by these fusion proteins was homogeneously conditional and reversible within 24–48 h. We used the regulable Abl:ER protein to study the kinetics of activation of Abl, and found a rapid (~10 min) or slower (over several hours) activation of Abl kinase activity as judged by phosphorylation of cellular substrates, depending on the absence or presence of the SH3 domain, respectively. We correlated the hormone-dependent activation of Abl with increased autophosphorylation, cytoplasmic localization, and the appearance of phosphotyrosine-containing structures at the plasma membrane.

Recently, we observed hormone-dependent formation of a 600–800 kDa complex containing Abl and several tyrosine phosphorylated proteins including p62 and p85 in sucrose gradients, which may correspond to these plasma membrane structures (P.Jackson, D.Baltimore, and D.Picard, unpublished results). For one identified substrate of activated Abl, the 85 kDa subunit of the phosphatidylinositol-3-kinase (PI-3-kinase), membrane association has been demonstrated to be important for activation of the associated PI-3-kinase activity (Varticovski *et al.*, 1991). We have also observed hormone-dependent coprecipitation of tyrosine phosphorylated proteins including p62, as observed for other transforming Abl proteins (Lugo *et al.*, 1990; Mayer *et al.*, 1992). Here, the Abl SH2 domain could promote complex formation by high affinity binding to tyrosine phosphorylated proteins, a function which appears to be essential for Abl transforming ability (Mayer *et al.*, 1990, 1992).

The c-Abl (IV):ER fusion protein localizes to the nucleus before activation with hormone, whereas upon hormone addition the *de novo* synthesized protein accumulates in the cytoplasm. This result is consistent with our observation that nuclear transport is the default condition for Abl in normal cells and that retention in the cytoplasm is regulated by the transformed state of the cells. In support of this idea, we have recently demonstrated that inducing Abl-dependent transformation can cause an epitope-tagged, nuclear, non-transforming variant of Abl to become retained in the cytoplasm (P.Jackson, D.Baltimore and R.Van Etten, unpublished).

The SH3-deleted Abl:ER fusion protein, ΔXB:ER, never goes to the nucleus and is more rapidly activated, consistent with immediate activation of the kinase without *de novo* synthesis. The failure of the SH3-deleted ΔXB:ER protein to translocate to the nucleus hints at a role for SH3 in modulating nuclear translocation. However, the unique perinuclear localization of and the presence of residual phosphotyrosine within the pool of ΔXB:ER protein in the absence of hormone suggest that this configuration may be

a special case, and not generally representative of normal or transforming Abl.

Different mechanisms for inactivation and activation of the Abl kinase?

Dominant inactivation by HBDs has been proposed to be due to the formation of a protein complex involving the heat-shock protein HSP90 and the protein fused to the HBD (for discussion see Picard *et al.*, 1988, 1990; Yamamoto *et al.*, 1988; and references therein). In the absence of hormone, HSP90, which is known to be bound hormone-reversibly to the HBD of steroid receptors, could interfere with various activities of the fusion polypeptide. Upon hormone addition, the release of HSP90 would relieve this inhibition. We suppose that the activated Abl kinase moiety of ΔXB:ER cannot function in the absence of hormone because HSP90, when bound to the HBD moiety, blocks access to substrate proteins or blocks another function essential for Abl-dependent fibroblast transformation, such as myristoylation-dependent membrane association or the SH2-phosphotyrosine binding function (Mayer *et al.*, 1990, 1992; Daley *et al.*, 1992). The fast kinetics (<10 min) and the lack of a protein synthesis requirement for activation of the ΔXB:ER fusion protein is consistent with a rapid release of HSP90 from such complexes.

In cIV:ER, the activation of the non-transforming Abl moiety by the HBD must involve a different mechanism as suggested by several observations: the higher concentrations of hormone required for activation, the different kinetics of morphological change, and the slower kinetics and the protein synthesis requirement for activation of the cIV:ER kinase. Since cIV:ER differs from ΔXB:ER only by the presence of SH3, this kinetic lag suggests that a distinct mechanistic step is required to overcome SH3-dependent negative regulation of Abl activity. The lag also correlates with and may be mechanistically related to the redistribution from the nucleus to the cytoplasm. We do not know whether there are differences in how HSP90 interacts with nuclear cIV:ER versus cytoplasmic ΔXB:ER fusion proteins and whether this explains differences in affinity for estradiol or the dose–response to estradiol for transformation.

Activation of Abl by fusion to proteins promoting oligomerization or by overexpression?

Two hypotheses for the activation of c-Abl by fusion to the ER HBD should be considered: (i) oligomerization and (ii) overexpression.

Oligomerization. The suggested ability of the HBD to promote hormone-dependent dimerization would provide a plausible mechanism for activation (Kumar and Chambon, 1988; Fawell *et al.*, 1990). Since transmembrane tyrosine kinases can be activated by oligomerization, non-receptor tyrosine kinases could be similarly regulated. This mechanism has indeed been suggested for activation of one such kinase, called c-Lck, in T-cell signaling (Bolen and Veillette, 1989). There is no direct biochemical evidence for dimerization/oligomerization of Abl, but others have observed activated versions of Abl in higher order complexes (Li *et al.*, 1988; Campbell *et al.*, 1990; Lugo *et al.*, 1990). The formation of a high molecular weight complex upon hormone addition has so far confounded our attempts to look directly for a hormone-dependent Abl dimer. However, the

possibility of Abl dimerization is supported by the ability of a kinase-active variant of Abl to transphosphorylate a kinase-defective variant in an *in vitro* kinase assay (Witte *et al.*, 1980). Further, when both variants are coexpressed in fibroblasts, the kinase active Abl can transphosphorylate the kinase inactive variant as assayed by anti-phosphotyrosine antibody (P.Jackson and R.Van Etten, unpublished data). An implication of the dimer hypothesis is that the fusion of sequences that promote oligomerization to the Abl protein should activate the Abl kinase activity. The ability of Gag and Bcr sequences found in naturally occurring transforming versions of Abl to dimerize or self-associate is consistent with this prediction (Yoshinaka *et al.*, 1984; Li *et al.*, 1988; Campbell *et al.*, 1990).

Overexpression. The ability of the wild type c-Abl (IV) kinase to be activated *in vivo* by vast overexpression has been demonstrated (Wang, 1988; Lugo *et al.*, 1990; Pendergast *et al.*, 1991). Typically, activation by overexpression has been seen only in transient expression systems and not in stably transfected murine fibroblast lines where such high levels of c-Abl overexpression are precluded by a 'toxic' or cytostatic effect of the c-Abl kinase. We speculate that the toxic effect of the Abl protein has been at least partially suppressed in the Abl:ER fusions, thus allowing overexpression to high levels. This idea is supported by our observation that the Abl:ER fusions are also conditional for the cytostatic effect of the Abl kinase (P.Jackson, D.Baltimore and D.Picard, in preparation). This property of the ER HBD to allow overexpression of 'toxic' proteins may be broadly useful.

Nonetheless, the p103^{cIV:ER} protein expressed in transformed NIH 3T3 cells was not more abundant than the cIV-Δ630 protein expressed in several non-transformed clonal lines (P.Jackson, unpublished data), suggesting that overexpression is not sufficient for transformation, and supporting the notion that the ER HBD has a dominant property causing Abl activation.

Application of regulation by HBDs

The extension of regulation-by-fusion to an HBD from transcription factors to kinases suggests that this strategy may provide regulable alleles for a variety of regulatory and enzymatic moieties, including gene products of unknown biochemical function. Indeed, other tyrosine and serine kinases have now been shown to be regulated as ER fusions (M.McMahon, personal communication). We have recently shown that the kinase activity of the Abl:ER fusion protein is also regulable in *Saccharomyces cerevisiae*, suggesting that the strategy may be generally applicable in eukaryotes (D.Picard and P.Jackson, unpublished). Steroid regulation may also be of considerable use in transgenic animals, especially in mammals, where homeostatic mechanisms generally preclude the use of temperature-sensitive mutants.

Materials and methods

Constructions

Constructs pPLcIV and pPLΔXB, encoding wild type or SH3-deleted versions of c-Abl (IV), respectively, have been described (Jackson and Baltimore, 1989). All new plasmids are based on these retroviral expression vectors.

pPLcIV:ER. A fragment encoding the HBD (amino acids 282–595) of the human ER, originally derived from plasmid HE14 (Kumar *et al.*, 1986), was fused to c-Abl (IV) (amino acids 1–630) at the *NarI* site following the sequence encoding the pentylsine nuclear localization signal. The HBD used in this study carries a point mutation (Gly to Val at amino acid 400), which lowers ligand affinity (Tora *et al.*, 1989). It is therefore less sensitive to spurious agonist activities in tissue culture medium.

pPLΔXB:ER. Identical to pPLcIV:ER except for the ΔXB deletion of the SH3 domain (Jackson and Baltimore, 1989).

pPLcIV-ΔN. A C-terminal deletion mutant of c-Abl (IV), derived from pPLcIV by inserting a *SpeI* linker in the unique *NarI* site, thus adding a nonsense mutation 3' to codon 630. This mutant retains the pentylsine nuclear localization signal.

pPLΔXB-ΔN. Identical to pPLcIV-ΔN, except for the ΔXB deletion of the SH3 domain.

pPLcIV(980):ER. The fragment encoding the HBD of ER was inserted into the unique *SalI* site of pPLcIV thus fusing c-Abl (IV) amino acids 1–980 to the HBD.

Cell culture and transfection

Transfection of NIH 3T3 cells and generation of clonal cell lines was as described by Jackson and Baltimore (1989). Briefly, 8×10^5 NIH 3T3 cells were cotransfected with 10 μg of plasmid DNA and 0.5 μg of a Moloney virus proviral clone, pZAP (Goff *et al.*, 1982), and scored for transformed foci. Populations of transformed cells were dislodged by squirting with medium, cloned to limiting dilution (≤ 30 positives per 96 well-plate) and screened for homogeneous hormone-dependent morphological transformation. Putative positive clones were expanded and verified by Western blotting, using the monoclonal antibody 19-84 which is specific for the Abl kinase domain (Schiff-Maker *et al.*, 1986). Independent transformed clones of a given mutant had very similar levels of expression. Immunofluorescence with anti-Abl or anti-ER sera was used to verify the homogeneity of the cloned lines.

Antisera, immunofluorescence and microscopy

Polyclonal rabbit serum specific for c-Abl (IV) was kindly given by Dr Owen Witte (UCLA). Polyclonal rabbit serum against the ER HBD was generously provided by Drs Steve Robbins and Mike Bishop (UCSF). Polyclonal rabbit antiserum was also generated against a GST–SH3 fusion protein (Cicchetti *et al.*, 1992). The SH3 serum was preadsorbed with GST protein and then affinity-purified against the GST–SH3 fusion protein. Both the anti-ER and anti-SH3 sera appeared monospecific for the highly overexpressed Abl:ER fusion proteins by Western blot analysis and SH3 staining could be blocked by including soluble SH3 protein. The anti-Abl monoclonal antibody, 19-84 (Schiff-Maker *et al.*, 1986), was affinity-purified on a goat anti-mouse IgG column from hybridoma supernatant (Cappel). The anti-phosphotyrosine monoclonal antibody, 4G10, was obtained commercially (UBI).

Immunofluorescence was performed as described by Van Etten *et al.* (1989). Briefly, cells grown on coverslips were fixed by methanol–acetone at -20°C , rehydrated, blocked in 5% normal donkey serum, stained with rabbit or mouse serum at 2 μg/ml, washed with PBS, stained with rhodamine-conjugated donkey anti-rabbit or mouse antibody (Jackson Immunoresearch) and counterstained with Hoechst 33358 (Sigma) to mark nuclei. Anti-ER HBD antiserum was used at a 1:300 dilution. Coverslips were mounted with Fluoromount G (Fisher), visualized on a Zeiss Axiophot or Nikon Diaphot, and photographed using TMAX 400 film (Kodak). Additional experiments using fixation with 4% paraformaldehyde and permeabilization with 0.5% Triton X-100 were performed to demonstrate that specific staining patterns were not an artefact of the fixation method. Time-lapse video microscopy was performed by growing cells on 25 mm coverslips and observing the cells under phase-contrast with a 40× air objective on a Zeiss ICM 405 with a CCD camera (Hamaguchi) and video recorder.

Hormone binding assay

The whole cell binding assay was as described by Taylor *et al.* (1984). ER HBD concentrations were determined by measuring hormone binding of whole cells in phenol-red free medium containing 0.1% bovine serum albumin (Sigma) and 1 nM [^3H]estradiol (NEN) in the presence or absence of 200 nM unlabeled β-estradiol. Affinity measurements were performed by incubating cells with increasing concentrations of radioligand (0–300 nM). Receptor concentrations and binding constants were deduced using a nonlinear iterative curve fitting program (Murlas *et al.*, 1982).

Western blot analysis and immunoprecipitation

Cell extracts were prepared in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 1% Triton, 0.1% SDS, 1% sodium deoxycholate, 1 mM Na₂VO₄, 40 mM NaF, 30 mM sodium pyrophosphate, 1 mM PMSF, 1% aprotinin, 10 µg/ml pepstatin) and clarified at 13 000 g for 15 min at 4°C. Lysates were boiled in sample buffer, run on 5–9% gradient SDS–polyacrylamide gels (50 µg protein for a 3 mm × 1 mm gel slot) and transferred to nitrocellulose or Immobilon (Millipore) for 500 mA·h. Filters were washed in Tris-buffered saline with 0.05% Tween 20 (TBST), blocked for 30 min in 1% BSA/TBST and incubated with affinity-purified anti-Abl or anti-phosphotyrosine antibodies at 2 µg/ml in TBST for 1 h. After three washes in TBST, filters were incubated with 0.5 µg/ml alkaline phosphatase (AP) conjugated donkey anti-mouse (Jackson Immunoresearch, Inc.) in TBST for 30 min. After three washes in TBST and one wash in AP buffer (100 mM Tris pH 9.5, 50 mM NaCl, 2.5 mM MgCl₂), filters were reacted in AP buffer plus 300 µg/ml nitroblue tetrazolium (NBT; Promega Biotech) and 150 µg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Promega Biotech). Filters were photographed using a yellow filter (Hoya).

Metabolic labeling and immunoprecipitations were as described by Jackson and Baltimore (1989).

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